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Caracterização biológica de extratos de própolis de diferentes regiões geográficas obtidos por Extração com Fluido Supercrítico e Extração Etanólica

Bruna Aparecida Souza Machado

São Cristóvão – SE

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Professora Orientadora: Dra. Francine Ferreira Padilha

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Caracterização biológica de extratos de própolis de diferentes regiões geográficas obtidos por Extração com Fluido Supercrítico e Extração Etanólica

TESE APRESENTADA AO PROGRAMA DE PÓS-GRADUAÇÃO EM BIOTECNOLOGIA – RENORBIO DA UNIVERSIDADE FEDERAL DE SERGIPE – UFS E UNIVERSIDADE TIRADENTES – UNIT, COMO PARTE DOS REQUISITOS NECESSÁRIOS PARA A OBTENÇÃO DO GRAU DE DOUTOR EM BIOTECNOLOGIA.

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DEDICATÓRIA

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"*Se enxerguei mais longe, foi porque me apoiei nos ombros de gigantes*".

(Isaac Newton)

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Minha vida é andar / Por esse país / Pra ver se um dia / Descanso feliz / Guardando as recordações / Das terras por onde passei / Andando pelos sertões / E dos amigos que lá deixei... (Luiz Gonzaga e Hervê Cordovil)

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RESUMO

A própolis é caracterizada como um material resinoso produzido pelas abelhas a partir da coleta de materiais de diferentes fontes vegetais e exibe propriedades farmacológicas e biológicas atribuídas à presença de diferentes classes de compostos fenólicos. A extração com fluido supercrítico (SFE) é uma operação que explora o poder de solvatação dos fluidos à temperatura e pressão acima de seus valores críticos para extrair ou separar componentes de uma amostra. Esta tecnologia tem se mostrado eficaz para obtenção de extratos a partir de matrizes naturais. O objetivo do estudo foi o de determinar as melhores condições de processo para a obtenção de extratos de própolis verde brasileira (Paraná) utilizando a SFE. Para isso, foi avaliada a influência de diferentes parâmetros, como S/F (massa de solvente – CO_2 – em relação à massa de soluto – própolis), percentual de co-solvente (1 e 2% de etanol), temperatura (40 e 50°C) e pressão (250, 350 e 400bar) utilizando dióxido de carbono (CO_2) supercrítico. Foi estudado o rendimento e obtidas as Isotermas de Rendimento Global (GYIs) e composição química dos extratos em relação ao teor de compostos fenólicos totais, flavonoides, atividade antioxidante e 3,5-diprenil-4-hidroxicinâmico (Artepillin C) e Ácido 4-hidroxicinâmico (ácido p-cumárico). Além disso, tendo em vista que as variações na composição química, e, consequentemente na atividade biológica da própolis estão associadas ao tipo e a sua origem geográfica, neste estudo, foram também avaliados extratos de própolis obtidos por extração supercrítica (SCO_2 – 50°C, 350bar, 1% de co-solvente e S/F de 110) e extração etanólica (EtOH – 80% a 70°C) de oito amostras de própolis de diferentes tipos, vermelha (Alagoas e Sergipe), verde (Minas Gerais e Paraná) e marrom (Paraná, Santa Catarina e Rio Grande do Sul), coletas em diferentes regiões geográficas do Brasil. As amostras foram caracterizadas em relação ao teor de proteínas, lipídeos, cinzas, minerais (sódio, potássio, cálcio e lítio), fibras, atividade de água e analisadas por Microscopia Eletrônica de Varredura (MEV). Os extratos foram analisados quanto ao teor de compostos fenólicos totais, flavonoides, atividade antioxidante in vitro (DPPH e ABTS), quantificação de Artepillin C e ácido p-cumárico por cromatografia líquida de alta eficiência (CLAE) e atividade antimicrobiana. Os extratos EtOH foram avaliados também quanto a atividade antiprolifefativa frente a linhagens de células B16F10 (murino). Em relação ao

emprego da SFE como método de obtenção de extratos de própolis (verde do Paraná) os melhores resultados foram identificados em 50°C e 350bar na presença de 1% de etanol (co-solvente) e S/F de 110. Nessas condições, foi identificado um teor de 8,93±0,01 e 0,40±0,05g/100g de Artepillin C e ácido p-cumárico, respectivamente, evidenciando a eficiência do processo de extração. Em relação à análise comparativa dos extratos EtOH e SCO_2 das diferentes amostras, as própolis vermelhas do nordeste brasileiro (Sergipe e Alagoas) apresentaram o maior potencial biológico, bem como, o maior conteúdo de compostos antioxidantes. Os melhores resultados foram apresentados para os extratos obtidos a partir da extração convencional (EtOH). Entretanto, foram identificadas as maiores concentrações de Artepillin C e ácido p-cumárico nos extratos SCO_2 (própolis verde e marrom), indicando assim uma maior seletividade do processo para extração destes compostos. Constatou-se que apesar do baixo rendimento do processo, os extratos obtidos por SFE apresentaram elevados teores de compostos de interesse, mostrando-se assim como um processo viável para obtenção de extratos de própolis verde. Os melhores resultados de atividade antioxidante foram apresentados pelas própolis vermelha e verde, evidenciando assim o potencial biológico dessas amostras. Por fim, destaca-se que a composição e atividade biológica da própolis brasileira variam significativamente a depender do tipo de amostra, da região geográfica de coleta e das condições de extração.

Palavras-chave: Extração supercrítica; Artepillin C; própolis; propriedades biológicas; origem geográfica; compostos fenólicos.

ABSTRACT

Propolis is a resinous material collected by bees from sprouts and barks of different vegetable source and the propolis has pharmacological and biological properties attributed to the presence of different classes of phenolic compounds. The supercritical fluid extraction (SFE) is an operation that explores the solvation power of fluids at temperature and pressure above its critical values, to extracting or separating components of a sample. This technology has been effective for obtaining extracts from natural matrices. The aim of the study was to define the best process conditions for obtaining Brazilian green propolis extracts (from Paraná) using the SFE. For that definition, the influence of different parameters was evaluated, such as S/F (solvent mass – CO_2 – relative to solute mass – propolis), percentage of co-solvent (1 and 2% ethanol), temperature (40 to 50°C) and pressure (250, 350 and 400bar) using supercritical carbon dioxide (CO_2). The Global Yield Isotherms (GYIs) were obtained through the evaluation of the yield, and the chemical composition of the extracts was also obtained in relation to the total phenolic compounds, flavonoids, antioxidant activity and 3,5-diprenil-4-hidroxicinamic (Artepillin C) and Acid 4-hidroxicinmic (p-coumaric acid). Furthermore, considering that the variations in the chemical composition, and consequently in the biological activity of propolis are associated with the type and geographical origin, samples of eight different propolis were evaluated, obtained by supercritical fluid extraction (SCO_2 - 50°C, 350bar, 1% co-solvent and S/F 110) and ethanoic extraction (EtOH - 80% at 70°C). The evaluated samples were: red (Alagoas and Sergipe), green (Minas Gerais and Paraná) and brown (Paraná, Santa Catarina and Rio Grande do Sul). The samples were characterized referring to protein, lipids, ash, minerals (sodium, potassium, calcium and lithium), fiber, water activity and analyzed by scanning electron microscopy (SEM). The extracts were analyzed for their content of phenolic compounds, flavonoids, in vitro antioxidant activity (DPPH and ABTS), quantification of Artepillin C and p-coumaric acid by high-performance liquid chromatography (HPLC) and antimicrobial activity. The EtOH extracts were evaluated as the anti-proliferative activity against B16F10 cell lines (murine) as well. For the SFE application as an extraction method to obtain propolis extract (green propolis from Paraná), the best results were found at 350bar, 50°C, presence of 1% ethanol (co-

solvent) and 110 S/F. Under these conditions, a content of 8.93±0.01 and 0.40±0.05g/100g Artepillin C and p-coumaric acid were identified, respectively, showing efficiency of the extraction process. Regarding to the comparative analysis of EtOH and SCO_2 extracts of different samples, the red propolis from northeastern Brazil (Sergipe and Alagoas) showed the highest biological potential and the highest content of antioxidant compounds. The best results were presented for the extracts obtained from the conventional extraction (EtOH). It was found that despite the low yield of the process, the extracts obtained by SFE showed elevated levels of compounds of interest, as well as proving to be a viable process to obtain propolis extracts. The best results of antioxidant activity were presented by red and green propolis, thus demonstrating the biological potential of these samples. Finally, there is the composition and biological activity of Brazilian propolis varies significantly depending on the sample type, geographic region gathering and extraction conditions.

Key words: Supercritical extraction; Artepillin C; propolis; biological properties; geographical origin; phenolic compounds.

1.0 Apresentação

Parte deste trabalho é oriundo do Projeto intitulado **"*Extração Supercrítica de Compostos Bioativos da Própolis*"** aprovado no Edital SENAI de Inovação (SENAI DN – Departamento Nacional) em parceria com o SENAI DR BA (Departamento Regional da Bahia) e com a empresa parceira Prodapys (Apis Nativa Produtos Naturais). O presente trabalho foi organizado no formato de capítulos para uma melhor apresentação e entendimento do mesmo, possuindo no total 8 (oito) capítulos. Cada capítulo refere-se a um item específico do trabalho, conforme descrito a seguir:

O **Capítulo 1** constitui a introdução geral da Tese. Neste capítulo são abordados, de forma resumida, vários aspectos relacionados a própolis, a aplicação científica e industrial de seus extratos, o potencial biológico dos diferentes tipos de própolis (verde, vermelha e marrom) e à justificativa para aplicação da técnica de extração supercrítica utilizando CO_2 como fluido supercrítico para obtenção de extratos de alto valor agregado dessa matriz natural.

O **Capítulo 2** constitui os objetivos da Tese. Neste capítulo é abordado o objetivo geral e detalhados os objetivos específicos do trabalho.

O **Capítulo 3** constitui o artigo de revisão publicado na *Revista Separation Science and Technology* intitulado de *Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives*. Neste capítulo são abordadas as principais aplicações da extração com fluido supercrítico através da busca em artigos científicos e patentes, onde os dados compilados e apresentados foram extraídos de 196 artigos científicos e 594 patentes. Além da descrição dos fundamentos e princípios da técnica de extração supercrítica, foram identificados os principais trabalhos desenvolvidos em diferentes áreas, como ciência de alimentos, produtos farmacêuticos, resíduos químicos, biocombustíveis e polímeros. Por fim, apresentou-se um estudo prospectivo com o perfil dos países detentores dessa tecnologia, evolução anual da mesma, e,

principais áreas de aplicação, bem como, as perspectivas futuras da tecnologia estudada.

O **Capítulo 4** constitui o artigo de revisão publicado na Revista *Sitientibus: Série Ciências Biológicas* intitulado de *Levantamento dos estudos com a própolis produzida no estado da Bahia*. Neste capítulo é apresentada a definição, aplicações biológicas e industriais da própolis, tipo de própolis a depender de sua origem geográfica, bem como, o panorama nacional da produção e importância econômica dessa matriz natural para o Brasil. São compilados os resultados identificados em 17 estudos que relatam especificamente o potencial da própolis produzida no estado da Bahia. Ressalta-se que a importância desse capítulo se deve principalmente porque nenhuma amostra de própolis originária da Bahia foi caracterizada experimentalmente no projeto que este trabalho encontra-se vinculado. Diante disso, foi possível levantar e mostrar as importantes aplicações e estudos já realizados com a própolis oriunda dessa região geográfica, identificando assim o potencial da própolis produzida nessa região.

O **Capítulo 5** constitui o artigo publicado na Revista *Plos One* intitulado de *Determination of parameters for the supercritical extraction of antioxidant compounds from green propolis using carbon dioxide and ethanol as co-solvent*. Neste capítulo foram determinadas as melhores condições de processo para obtenção de extrato de própolis verde brasileira utilizando a tecnologia de extração supercrítica. A partir dos resultados de caracterização encontrados para os extratos obtidos nas diferentes condições de processo, foi possível identificar os valores de temperatura, pressão, relação de massa de solvente por massa de amostra (S/F) e percentual de co-solvente para obtenção de extratos com maior potencial biológico utilizando CO_2 como fluido supercrítico no processo extrativo.

O **Capítulo 6** constitui o artigo aceito para publicação na Revista Plos One intitulado de *Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil*. Neste capítulo foram avaliados

extratos de própolis obtidos por extração supercrítica e extração etanólica de oito amostras de própolis de diferentes tipos, vermelha, verde e marrom, coletas em diferentes regiões do Brasil, mostrando assim que as variações na composição química, e consequentemente, na atividade biológica da própolis estão inteiramente associadas ao tipo e a sua origem geográfica, bem como, ao método de extração. Ressalta-se que os parâmetros de processo utilizados para a obtenção dos extratos utilizando a tecnologia de extração supercrítica foram retirados do capítulo anterior.

No **Capítulo 7** é apresentado as considerações finais do presente trabalho.

Por fim, no **Capitulo 8** está listada toda a produção científica e técnica desenvolvida durante do período do doutorado, bem como, os prêmios obtidos.

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Capítulo 1. Introdução Geral

2.0 Introdução

A própolis é uma substância resinosa coletada pelas abelhas (*Apis mellifera* L.) a partir de brotos e folhas de diferentes árvores e plantas, misturado com pólen, bem como enzimas secretadas pelas próprias abelhas (Marcucci, 1995; Bankova et al., 2000; Choi et al., 2006). É considerada como um remédio popular tradicional e foi submetida a vários estudos farmacológicos e químicos nos últimos 30 anos (Zordi et al., 2014; Andreu et al., 2015). Essa matriz é amplamente utilizada em formulações cosméticas e produtos farmacêuticos, sendo um dos produtos naturais mais conhecidos e utilizados (Costa et al., 2013; Machado e Padilha, 2014). Muitos trabalhos relacionados com as suas propriedades terapêuticas, bem como com as diferenças nas suas composições foram publicadas até agora (Lotfy, 2006; Sforcin et al., 2011; Kasote et al., 2015; Cottica et al., 2015).

Atualmente, os novos métodos de extração estão sendo investigados para substituir os métodos clássicos, como por exemplo, a extração com solvente, maceração, destilação a vácuo entre outros. Um dos mais promissores métodos é a extração com fluidos supercríticos (*Supercritical Fluid Extraction* – SFE), especificamente com o uso de dióxido de carbono (CO_2) como o fluido supercrítico (Machado et al., 2013; Machado et al., 2014). Esta tecnologia tem se mostrado eficaz para aplicações em processos químicos, petroquímicos, farmacêuticos, ambientais e alimentícios principalmente por ser considerada uma tecnologia limpa. Além disso, o CO_2 é considerado, não explosivo, não tóxico, disponível em alta pureza e com baixo custo (Lucas et al., 2002; Ghoreishi et al., 2011; Bashipour e Ghoreishi, 2014). A SFE é capaz de manter as propriedades antioxidantes dos extratos obtidos devido à utilização de baixas temperaturas, e esta característica é de extrema importância para as indústrias farmacêuticas e de alimentos (Ge et al., 2002; Agostini et al., 2012; Bashipour et al., 2012; Machado et al., 2013). Torna-se importante destacar que embora os fluidos supercríticos tenham sido conhecidos há mais de um século, estes ainda estão sendo tratados como uma descoberta recente (Machado et al., 2013). A

utilização desta técnica de extração como instrumento analítico e preparativo somente ganhou destaque nos últimos 30 anos, o que é confirmado pelo elevado número de artigos publicados e patentes depositadas nesta área em todo o mundo.

A aplicação da SFE com a utilização de CO_2 supercrítico para a extração de compostos de interesse da própolis é bastante viável. Zordi et al., (2014) avaliaram a influência de diferentes parâmetros para a extração de compostos lipofílicos de própolis italianas utilizando CO_2 supercrítico. Os resultados obtidos indicaram duas possíveis aplicações do CO_2 supercrítico para a extração de própolis: obter frações lipofílicas enriquecidas em componentes específicos ou como pré-tratamento do material bruto para facilitar extração adicional com etanol. Biscaia et al. (2009) compararam os rendimentos de extração de própolis brasileiras obtidos por diferentes processos, ou seja, a extração por SFE em uma única fase e em duas fases, com CO_2 e CO_2 acrescido de co-solvente, bem como por Soxhlet e maceração com diferentes solventes. Foi identificado que o uso de etanol como co-solvente no processo de SFE aumentou o rendimento da extração em cerca de três vezes, em comparação com a extração apenas com CO_2.

Um importante ácido fenólico presente em amostras de própolis brasileiras e já relatadas em diferentes estudos é o 3,5-diprenil-4-ácido hidroxicinâmico (HPPC), também conhecido como Artepillin C (Keshavarz et al., 2009; Paviani et al., 2010; Cheung et al., 2011; Paviani et al., 2012). Lee et al., (2007) investigaram individualmente solventes orgânicos e CO_2 supercrítico nos processos de extração para recuperar o Artepillin C da própolis brasileira. Alguns estudos apontam importantes atividades biológicas associadas ao Artepillin C, principalmente seus efeitos antitumorais (Matsuno et al., 1997; Kimoto et al., 1998; Shimizu et al., 2005). Foram relatados os efeitos inibidores de Artepillin C em carcinoma renal (Kimoto et al., 2000), supressão de angiogênese induzida por tumor (Ahn et al., 2007), efeitos anti-inflamatórios em edema de pata em camundongos (Paulino et al., 2008), efeitos anti-leucemicos (Kimoto et al. 2001a) e a inibição no desenvolvimento de cancro pulmonar (Kimoto et al. 2001b).

A própolis brasileira é altamente valorizada no mercado internacional e ganhou enorme importância comercial devido a uma grande variedade de benefícios a saúde já comprovados (Salatino et al., 2011; Machado et al., 2012). A própolis verde, marrom

e vermelha são os tipos mais comuns de própolis brasileira. A própolis verde é oriunda da espécie vegetal *Baccharis dracunculifolia* e rica em compostos fenilpropanoides prenilados, triterpenoides e ácidos benzoicos e clorogênicos (Righi et al., 2011). A própolis marrom é derivada de espécies de *Copaifera* e contém principalmente flavonoides e terpenos (Sawaya et al., 2006). A própolis vermelha brasileira é obtida a partir de *Dalbergia ecastophyllum* e contém isoflavonas, benzofenonas preniladas entre outros compostos (Trusheva et al., 2006; Piccinelli et al., 2011; Kasote et al., 2014).

A composição química da própolis é conhecida por ser complexa e variável entre as estações e regiões (Salatino et al., 2011). Numerosos fatores, tais como a composição florística da área, localização e tempo de coleta possuem relação com a composição química da própolis (Tagliacollo e Orsi, 2011; Kasote et al., 2014), e consequentemente com os seus efeitos biológicos. Por causa das diferenças geográficas, as amostras de própolis da Europa, América do Sul ou Ásia têm diferentes composições químicas (Marcucci, 1995). Diferentes estudos têm avaliado a composição química e atividades biológicas de amostras de própolis coletadas em regiões distintas. Por exemplo, as atividades biológicas de extratos de própolis da Coreia foram examinadas para a avaliação da qualidade em comparação com a do Brasil por Choi et al., (2006). As atividades antibacterianas e antioxidantes de própolis de diferentes procedências da Argentina foram investigadas e correlacionadas os valores com os níveis totais de compostos polifenólicos e flavonoides (Chaillou e Nazareno, 2009). As amostras analisadas apresentaram uma notável variabilidade em sua atividade antioxidante e poucas diferenças na atividade antibacteriana frente às cepas estudadas. A composição química, atividade antioxidante *in vitro* e a atividade antimicrobiana do extrato etanólico de doze amostras de própolis da Grécia continental, ilhas gregas, e no leste Chipre foram determinadas por Kalogeropoulos et al. (2009). Foi identificado que apesar das diferenças na composição química das amostras de própolis de diferentes localizações geográficas, todas apresentaram atividades antibacterianas e antifúngicas significativas.

Por fim, destaca-se que diante das propriedades antioxidantes e antimicrobianas, associado ao fato de que vários dos seus componentes já estão presentes em alimentos e/ou aditivos alimentares, sendo essencialmente

reconhecidos como GRAS (*Generally Recognized as Safe*) (Burdock, 1998), a própolis é considerada como uma matéria-prima atraente para ser utilizada como um conservante natural em novas aplicações alimentares. Isso atende a demanda de antioxidantes e antimicrobianos naturais, impulsionadas pela crescente sensibilização dos consumidores por alimentos naturais e minimamente processados, com conservantes tradicionais ausentes ou em concentrações muito baixas (Han e Park, 1995; Tosi et al., 2007; Costa et al., 2014). Além disso, a elevada utilização de solventes orgânicos nos diferentes processos industriais representa um problema discutido globalmente devido aos danos causados ao meio ambiente.

Neste contexto, a justificativa deste trabalho baseia-se em três pontos principais: (1) identificar as condições de processos ideais para obtenção de extratos de própolis com elevados teores de compostos biologicamente ativos utilizando uma tecnologia limpa (extração com fluido supercrítico), é de grande importância e interesse para as indústrias de alimentos, farmacêutica e cosmética, tendo em vista o potencial de aplicação destes extratos em novos produtos; (2) conhecer o perfil químico e biológico de amostras de própolis do Brasil de diferentes tipos e origens geográficas é de grande relevância para a comunidade científica e industrial, pois além de contribuir para a valorização desta matéria-prima revela o potencial e características das mesmas; e ainda (3) realizar uma análise comparativa correlacionando o método de extração, tipo de amostra, caracterização química e caracterização biológica contribui para posicionar estudos futuros ou desenvolvimento de novos produtos utilizando o método mais eficiente para obtenção de extratos a depender do seu tipo de aplicação. Destaca-se por fim que são escassos os estudos que avaliam comparativamente diferentes tipos de própolis brasileira, e ainda, que utilizem métodos distintos para a obtenção dos extratos.

Referências

AHN, M. R.; KUNIMASA, K.; OHTA, T.; KUMAZAWA, S.; KAMIHIRA, M.; KAJI, K.; UTO, Y.; HORI, H.; NAGASAWA, N.; NAKAYAMA, T. Suppression of tumor-induced angiogenesis by Brazilian propolis: Major component artepillin C inhibits in vitro tube formation and endothelial cell proliferation. *Cancer Letters*, v. 252, n. 2, p. 235–243, 2007.

AGOSTINI, F.; BERTUSSI, R. A.; AGOSTINI, G.; SANTOS, A. T. A.; ROSSATO, M.; VANDERLINDE, R. Supercritical extraction from vinification residues: fatty acids, α-tocopherol, and phenolic compounds in the oil seeds from different varieties of grape. *Scientific World Journal*, v. 2012, p. 1–9, 2012.

ANDREU, G. L. P.; REIS, F. H. Z.; DALALIO, F. M.; FIGUEREDO, Y. N.; RUBIO, O. C.; UYEMURA, S. A.; CURTI, C.; ALBERICI, L. C. The cytotoxic effects of brown Cuban propolis depend on the nemorosone content and may be mediated by mitochondrial uncoupling. *Chemico-Biological Interactions*, v 228, p. 28-34, 2015.

BANKOVA, V. S.; CASTRO, S. L. D.; MARCUCCI, M. C. Propolis: Recent advances in chemistry and plant origin. *Apidologie*, v. 31, n. 1, p. 3–15, 2000.

BASHIPOUR, F.; GHOREISHI, S. M. Experimental optimization of supercriticalextraction of ß-carotene from *Aloe barbadensis Miller* via genetic algorithm. *The Journal of Supercritical Fluids*, v. 72, p. 312–319, 2012.

BASHIPOUR, F.; GHOREISHI, S. M. Response surface optimization of supercritical CO_2 extraction of α-tocopherol from gel and skin of *Aloe vera* and almond leaves. *The Journal of Supercritical Fluids*, v. 95, p. 348–354, 2014.

BISCAIA, D.; FERREIRA, S. R. S. Propolis extracts obtained by low pressure methods and supercritical fluid extraction. *The Journal of Supercritical Fluids*, v. 51, n. 1, p. 17–23, 2009.

BURDOCK, G. A. Review of the biological properties and toxicity of bee propolis (propolis). *Food Chemistry and Toxicology*, v. 36, p. 347–363, 1998.

CHAILLOU, L. L.; NAZARENO, M. A. Bioactivity of propolis from Santiago del Estero, Argentina, related to their chemical composition. *LWT - Food Science and Technology*, v. 42, n. 8, p. 1422–1427, 2009.

CHEUNG, K. W.; SZE, D. M. Y.; CHAN, W. K.; DENG, R. X.; TU, W.; CHAN, G. C. F. Brazilian green propolis and its constituent, Artepillin C inhibits allogeneic activated human CD4 T cells expansion and activation. *Journal of Ethnopharmacology*, v. 138, n. 2, p. 463-471, 2011.

CHOI, Y. M.; NOH, D. O.; CHO, S. Y.; SUH, H. J.; KIM, K. M.; KIM J. M. Antioxidant and antimicrobial activities of propolis from several regions of Korea. *Food Science and Technology*, v. 39, n. 7, p. 756–761, 2006.

COSTA, A. S.; MACHADO, B. A. S.; UMSZA-GUEZ, M. A.; CIRQUEIRA, M. G.; NUNES, S. B.; PADILHA, F. F. Survey of studies with propolis produced in the state of Bahia, Brazil. *Sitientibus: Série Ciências Biológicas*, v. 13, p. 1-7, 2013.

COSTA, S. S.; MACHADO, B. A. S.; SOUZA, C. O.; DRUZIAN, J. I.; GUIMARÃES, A. G. Bi-Functional biobased packing of the cassava starch, glycerol, licuri nanocellulose and red propolis. *Plos One*, v. 9, n. 11, p. e112554-e112554, 2014.

COTTICA, S. M.; SABIK, H.; ANTOINE, C.; FORTIN, J.; GRAVELINE, N.; VISENTAINER, J. V.; BRITTEN, M. Characterization of Canadian propolis fractions obtained from two-step sequential extraction. *LWT - Food Science and Technology*, v. 60, n. 1, p. 609-614, 2015.

GHOREISHI, S. M.; BATAGHVA, E. Supercritical extraction of evening primrose oil: experimental optimization via response surface methodology. *American Institute of Chemical Engineers Journal*, v. 57, p. 3378–3384, 2011.

HAN, S. K.; PARK, H. K. A study on the preservation of meat products by natural propolis: Effect of EEP on protein change of meat products. *Korean Journal of Animal Science*, v. 37, p. 551–557, 1995.

KESHAVARZ, M.; MOSTAFAIE, A.; MANSOURI, K.; SHAKIBA, Y.; MOTLAGH, H. R. M. Inhibition of Corneal Neovascularization with Propolis Extract. *Archives of Medical Research*, v. 40, n. 1, p. 59-61, 2009.

KALOGEROPOULOS, N.; KONTELES, S. J.; TROULLIDOU, E.; MOURTZINOS, I.; KARATHANOS, V. T. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. *Food Chemistry*, v. 116, n. 2, p. 452–461, 2009.

KASOTE, D.; SULEMAN, T.; CHEN, W.; SANDASI, M.; VILJOEN, A.; VUUREN, S. V. Chemical profiling and chemometric analysis of South African propolis. *Biochemical Systematics and Ecology*, v. 55, p. 156-163, 2014.

KASOTE, D.; AHMAD, A.; CHEN, W.; COMBRINCK, S.; VILJOEN, A. HPTLC-MS as an efficient hyphenated technique for the rapid identification of antimicrobial compounds from propolis. *Phytochemistry Letters*, v. 11, p. 326-331, 2015.

KIMOTO, T.; ARAI, S.; KOHGUCHI, M.; AGA, M.; NOMURA, Y.; MICALLEF, M. J.; KURIMOTO, M.; MITO, K. Apoptosis and suppression of tumor growth by Artepillin C

extracted from Brazilian propolis. *Cancer Detection and Prevention*, v. 22, p. 506-515, 1998.

KIMOTO, T.; AGA, M.; HINO, K.; KOYA-MIYATA, S.; YAMAMOTO, Y.; MICALLEF, M. J.; HANAYA, T.; ARAI, S.; IKEDA, M.; KURIMOTO, M. Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. *Anticancer Research*, v. 21, p. 221–228, 2001a.

KIMOTO, T.; KOYA-MIYATA, S.; HINO, K.; MICALLEF, M. J.; HANAYA, T.; ARAI, S.; IKEDA, M.; KURIMOTO, M. Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and Artepillin C. *Virchows Archiv*, v. 438, p. 259–270, 2001b.

LEE, Y.-N., CHEN, C.-R., YANG, H.-L., LIN, C.-C., CHANG, C.-M. J. Isolation and purification of 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) in Brazilian propolis by supercritical fluid extractions. *Separation and Purification Technology*, v. 54, p. 130-138, 2007.

LOTFY, M. Biological Activity of Bee Propolis in Health and Disease. *Asian Pacific Journal of Cancer Prevention*, v. 7, p. 22-31, 2006.

LUCAS, A.; OSSA, E. M. D-L.; RINCÓN, J.; BLANCO, M. A.; GRACIA, I. Super-critical fluid extraction of tocopherol concentrates from olive tree leaves. The Journal of Supercritical Fluids, v. 22, p. 221–228, 2002.

MACHADO, B. A. S.; CRUZ, L. S; NUNES, S. B.; UMSZA-GUEZ, M. A.; PADILHA, F. F. Estudo prospectivo da própolis e tecnologias correlatas sob o enfoque em documentos de patentes depositados no Brasil. *GEINTEC - Gestão, Inovação e Tecnologias*, v. 2, p. 221-235, 2012.

MACHADO, B. A. S.; PEREIRA, C. G.; NUNES, S. B.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives. *Separation Science and Technology*, v. 48, n. 3, p. 2741-2760, 2013.

MACHADO, B. A. S.; PADILHA, F. F.; NUNES, S. B.; GUEDES, C. M. C.; COSTA, A. S.; UMSZA-GUEZ, M. A.; SILVA, R. P. D. Process for obtaining 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) propolis extract using supercritical carbon dioxide and co-solvent. 2014. Patente brasileira BR1020140320121.

MACHADO, B. A. S.; PADILHA, F. F. O processo de proteção das Indicações Geográficas no Brasil. In: SANTOS, F. L. (Org.). *Desenvolvimento e perspectivas da propriedade intelectual no Brasil*. Cruz das Almas: Editora UFRB, 2014, p. 105-120.

MARCUCCI, M. C. Propolis: Chemical composition, biological properties and therapeutic activity. *Apidologie*, v. 26, p. 83–99, 1995.

PAULINO, N.; ABREU, S. R. L.; UTO, Y.; KOYAMA, D.; NAGASAWA, H.; HORI, H.; DIRSCH, V. M.; VOLLMAR, A. M.; SCREMIN, A.; BRETZ, W. A. Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. *European Journal of Pharmacology*, v. 587, n. 1–3, p. 296–301, 2008.

PAVIANI, L. C.; DARIVA, C.; MARCUCCI, M. C.; CABRAL, F. A. Supercritical carbon dioxide selectivity to fractionate phenolic compounds from the dry ethanolic extract of Propolis. *Journal of Food Process Engineering*, v. 33, p. 15–27, 2010.

PAVIANI, L. C.; DARIVA, C.; MARCUCCI, M. C.; SÁNCHEZ-CAMARGO, A.; CABRAL, F. A. Supercritical CO_2 extraction of raw propolis and its dry ethanolic extract. *Brazilian Journal of Chemical Engineering*, v. 29, 243-251, 2012.

PICCINELLI, A. L.; LOTTI, C.; CAMPONE, L.; CUESTA-RUBIO, O.; FERNANDEZ, M. C.; RASTRELLI, L. Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography photodiode array detection/electrospray ionization tandem mass Spectrometry. *Journal of Agricultural and Food Chemistry*, v. 59, p. 6484-6491, 2011.

RIGHI, A. A.; ALVES, T. R.; NEGRI, G.; MARQUES, L. M.; BREYER, H.; SALATINO, A. Brazilian red propolis: unreported substances, anti-oxidant and antimicrobial activities. *Journal of the Science of Food and Agriculture*, v. 91, p. 2363-2370, 2011.

SALATINO, A.; FERNANDES-SILVA, C. C.; RIGHI, A. A.; SALATINO, M. L. F. Propolis research and the chemistry of plant products. *Natural Product Reports*, v. 28, n. 5, p. 925-936 2011.

SAWAYA, A. C. H. F.; CUNHA, I. B. S.; MARCUCCI, M. C.; RODRIGUES, R. F. D.; EBERLIN, M. N. Brazilian propolis of *Tetragonisca angustula* and *Apis mellifera*. Apidologie, v. 37, p. 398-407, 2006.

SHIMIZU, K.; SWADESH, K. D.; HASHIMOTO, T.; SOWA, Y.; YOSHIDA, T.; SAKAI, T.; MATSUURA, Y.; KANAZAWA, K.; Artepillin C in Brazilian propolis induces G0/G1 arrest

via stimulation of Cip1/p21 expression in human colon cancer cells. *Molecular Carcinogenesis*, v. 44, p. 293–299, 2005.

SFORCIN, J. M.; BANKOVA, V. Propolis: is there a potential for the development of new drugs?

Journal of Ethnopharmacology, v. 133, p. 253–260, 2011.

TAGLIACOLLO, V. A.; ORSI, R. O. Quality of propolis commercialized in the informal market. *Ciência e Tecnologia Alimentos*, v. 31, p. 752-757, 2011.

TRUSHEVA, B.; POPOVA, M.; BANKOVA, V.; SIMOVA, S.; MARCUCCI, M. C.; MIORIN, P. L.; PASIN, F. R.; TSVETKOVA, I. Bioactive constituents of Brazilian red propolis. *Evidence-Based Complementary and Alternative Medicine*, v. 3, p. 249-254, 2006.

TOSI, E. A.; RÉ, E.; ORTEGA, M. E.; CAZZOLI, A. F. Food preservative based on propolis: Bacteriostatic activity of propolis polyphenols and flavonoids upon *Escherichia coli*. *Food Chemistry*, v. 104, p. 1025–1029, 2007.

ZORDI, N.; CORTESI, A.; KIKIC, I.; MONEGHINI, M.; SOLINAS, D.; INNOCENTI, G.; PORTOLAN, A.; BARATTO, G.; DALL'ACQUA, S. The supercritical carbon dioxide extraction of polyphenols from propolis: a central composite design approach. The Journal of Supercritical Fluids, v. 95, p. 491–498, 2014.

Capítulo 2. Objetivos

3.0 Objetivos

3.1 Objetivo Geral

O objetivo geral deste estudo foi investigar e determinar condições de processo de extração, incluindo temperatura, pressão, percentual de co-solvente e S/F (massa de solvente/massa de soluto) para obtenção de compostos de interesse presentes na própolis verde através da tecnologia de extração supercrítica, utilizando dióxido de carbono como solvente. Além disso, obter, caracterizar e avaliar comparativamente extratos de oito amostras de própolis oriundas de diferentes regiões geográficas do Brasil (verde, vermelha e marrom) por extração supercrítica (condições selecionadas) e extração convencional (etanólica).

3.2 Objetivos Específicos

Como objetivos específicos deste estudo, destaca-se:

(1) Elaborar um estudo prospectivo e de revisão utilizando informações científicas e tecnológicas contidas nas bases de artigos e patentes sobre a tecnologia de extração supercrítica, identificando os principais trabalhos desenvolvidos em diferentes áreas, como ciência de alimentos, produtos farmacêuticos, resíduos químicos, biocombustíveis e polímeros, bem como, apresentar a evolução anual do depósito de patentes (até 2013) e principais países e empresas depositantes;

(2) Elaborar um estudo de revisão a partir do levantamento de trabalhos publicados em bases de dados de artigos científicos e patentes relacionados a própolis produzida no estado da Bahia, correlacionando os aspectos físico-químicos, as atividades biológicas e aplicações;

(3) Definir as condições de extração supercrítica para a própolis verde e obtenção de sua respectiva composição em relação ao teor de: (i) Artepillin C (3,5-diprenil-4-

hidroxicinâmico), (ii) ácido p-cumárico (ácido 4-hidroxicinâmico), (iii) compostos fenólicos totais, (iv) flavonoides totais e (v) atividade antioxidante (DPPH - 2,2-difenil-1-picrilhidrazila);

(4) Comparar extratos de oito amostras de própolis (verde, vermelha e marrom) oriundas de diferentes regiões geográficas do Brasil, obtidos por: (i) extração com fluido supercrítico – CO_2 (condições pré-selecionadas) e (ii) extração etanólica (convencional) em relação ao teor de: (i) compostos fenólicos totais, (ii) flavonoides totais, (iii) atividade antioxidante (DPPH e ABTS - 2,2'-azino-bis-[3-etilbenzotiazolina-6-acido sulfônico]), (iv) atividade antimicrobiana, (v) atividade antitumoral *in vitro* frente a células de B16F10 (murino) e (vi) composição química frente a alguns marcadores de interesse;

(5) Caracterizar as amostras brutas de própolis (verde, vermelha e marrom) oriundas de diferentes regiões geográficas quanto ao teor de: (i) proteínas, (ii) lipídeos totais, (iii) cinzas, (iv) minerais, (v) atividade de água, (vi) umidade, (vii) fibras e (viii) morfologia.

Capítulo 3. Artigo

4.0 CAPÍTULO 3: Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives

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Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives

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The main objective of this review is to approach and highlight the main applications of Supercritical Fluid Extraction (SFE) through the search in scientific papers and patents. The principles of the technique and the identification of the main works developed in different areas, such as food science, pharmaceuticals, chemical residues, biofuel, and polymers are presented. Additionally, it presents the new and innovative combined methods where SFE has been used, as well as the perspectives of this technology and the profile of countries that own the rights for the use of SFE through patent registration. In this review a compilation of the data extracted from 196 different scientific article and 594 patent documents, from a universe of 2.314 documents was done, showing the improvement of the technique in the last years.

Keywords food; natural compounds; supercritical extraction patents; supercritical fluid extraction

INTRODUCTION

Extraction with supercritical fluid (SFE) is an operation that explores the solvation power of the fluids at pressure and temperature above its critical value, in order to extract or separate components of a sample. The application of SFE is based on the experimental observation that many gases increase their dissolution power when compressed above a critical point (1,2).

The importance of using these fluids started to be disseminated at the end of the twentieth century. In 1879, Hannay and Hogard proposed a model where the simultaneous increase in pressure and temperature of certain chemical substances increased its solubility. In 1906, Buncher (3) confirmed this model through experiments, better systematizing the process. Bundschuh, in 1986 (4), verified that the fluids, in these conditions, have their properties (density, diffusion, and viscosity) simultaneously show gas and liquid characteristics.

Only through the second half of the twentieth century did these fluids started to be applied in industrial scale, adopting initially the name of dense gases, pressurized fluids, and finally, supercritical fluids (3,5). One of the aspects responsible for the delay in using this technology was the difficulty in devising safe equipments to operate in high temperatures and especially at high pressure, depending on the type of fluid used and the problem that has been overcome in the past years.

Even though the supercritical fluids have been known for over a century, they are still treated as a recent finding. The utilization of this extraction technique as an analytical and preparative instrument only acquired relevance in the past 25 years, which is confirmed by the high number of published papers and patents deposited in this area throughout the whole world.

The high utilization of organic solvents in the different industrial processes, such as fat and oil extraction, obtaining bioactive functional compounds, removal of heavy metals, polymer processing, fuel production, among others, represent a globally discussed issue, due to the harm caused to the environment. In light of this picture, in 1987, the Montreal Protocol was introduced, and in 1997 the Kyoto Protocol, which had as the main objective to restrict or eliminate the production and utilization of solvents that cause harm to the ozone layer (6). The great interest of the scientific community and the industrial sector for SFE is directly related to the restrictions to the use of organic solvents, both in the preparative processes of samples

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used in the various industries, and in a higher ecological consciousness in the use of different analysis methods involving extraction.

Presently, the utilization of SFE is extensively applied not only to the food and drug areas, but also in the areas of toxicology, chemistry, environment, textile, petrochemical, polymers, among others (7–10). Supercritical fluids show low viscosity as a gas, high density as liquids, and intermediate diffusion between gases and liquids, varying with its density (11,12). They are easily adaptable to the various separation processes, which involve wide levels of temperature and pressure. Such characteristic allows supercritical fluids to be used to separate technically unstable materials (oils and polyunsaturated fats acids, vitamins, carotenoids, antioxidant compounds) at low temperatures, and even realize separations with small variations of pressure, due to the high compressibility and exponential solubility (13). These characteristics turn these fluids adequate as substitutes to organic solvents in extraction processes.

The SFE has advantages and limitations when compared to the classic extraction techniques, such as the liquid-liquid extraction, maceration, and extraction with organic solvent (14,15). One of the main advantages of the utilization of SFE is the use of carbon dioxide (CO_2) as an extractor solvent, since this is a substance that has highly superior properties than the majority of the organic solvents (hexane, pretroleum ether, tolueno, chloroform, methanol, ethanol). There is a great variety of compounds that can be used as supercritical solvents, such as water, benzene, toluene, ethane, ethanol, among others. CO_2 is the most used solvent, especially due to its low cost characteristics, and the fact that it is easily available in high purity form, it is non-toxic, non-inflammable, and non-explosive. Another advantage is that the CO_2 is a gas at room temperature and pressure, which makes it easy to be removed after the extraction process (16). However, it is important to point out that the main limitation of the SFE utilization is linked to the high cost of the commercially available equipment when compared to the conventional extraction systems.

The SFE using CO_2 is frequently applied in food and natural sample preparation due to its capacity to be operated in relatively low temperatures, using a non-oxidative medium, which allows the extraction of thermally unstable or easily oxidative compounds. Besides that, the extraction can be facilitated by the addition of low quantities of other compounds, called co-solvents or modifiers, such as water and ethanol, which alter the solvation power of the supercritical CO_2 when it cannot extract more polar compounds (14,17). This flexibility allows the adequate extraction conditions for the specific needs of the products to be extracted and for the final desired product.

Various studies have demonstrated the many advantages of using supercritical fluids, especially CO_2, in order to extract different types of substances in a wide variety of sources (matrix). In that sense, some works have recently been published, focused on the evaluation of the economical and industrial feasibility of certain processes developed with the utilization of SFE in the pharmacological area, such as the extraction of fennel, rosemary, and anise essential oils (18), valerian (19), and bioactive substances of propolis (20,21). In the area of food, the extraction of antioxidant substances, such as tomato lycopene, should be emphasized (22–25) and mangosteen xanthones (*Garcinia mangostana*) (26) besides the extraction and fractionating of carbohydrates (27).

In chemistry, SFE has been used in polymer processing, using supercritical CO_2 as a substitute of organic solvents in the modification, composite formation, production of micro cellular foam particles, and polymerization (28,29). It has also been used in the removal of heavy metals from different types of samples (water, soil, tissues, wood) (30–32), in the area of bio-fuel production, in obtaining seed oils with the utilization of supercritical ethanol in the process of transesterification (33,34), and in the production of bioethanol through the treatment of sugar cane bagasse from the cellulose matrix, breaking into fermentable sugars using the supercritical CO_2 (35,36).

In the past years, the questions related to intellectual property, particularly the patents requests, have resulted in the interest of both the developed countries, holders of the innovation tools, and of countries that intend to assume a competitive role in an increasingly globalized market. The interest and importance given to SFE can also be confirmed through the increase in the number of patents deposited by companies and universities of different countries, such as China, United States, Japan, South Korea, members of the European Union, Canada, among others, with the objective to protect the new technological advances of this technique. From the research about innovations in the area of SFE, it is possible to identify new fields for the application of the technique, which has been modernized and therefore more adequate to various areas and industrial applications. China and the United States are examples of countries that invest in applied research about SFE, since they are the biggest owners of such technology.

A large part of the importance given to SFE is closely linked to the need to substitute the organic solvents, in the face of the international campaigns enforced by the environmentalists, and also the tendency for a fast increase of the industrial costs due to the high energy costs derived from the petroleum crisis. Therefore, SFE has become an important alternative process widely used in the extraction of materials in scientific and industrial scale. In view of the great importance in the utilization of such technique as an extracting, preparative, and analytical method for various substances, the main objective of the present review is to highlight the main areas for SFE application, the principles

of this technique, as well as to identify the main works developed in this area through research of scientific article databases, and finally, to present a prospective study about the technological tendencies of SFE, through research in patent databases. Besides that, the aim is to identify new application fields for this technique, relating it to future perspectives.

METHODOLOGICAL ASPECTS

For the elaboration of this review about SFE, the methodology used involved research in patents and scientific papers databases. The relevant works, that is, those which described the SFE as a preparative, extracting, or analytical method for food samples, obtainment of bioactive and/or functional compounds from natural matrix, and new methods for application of SFE, were selected and discussed in this review as well as exposed in tables organized by areas.

The patent documents that mentioned the relevant protected technology were selected by area of application, date of deposit, and country of origin of the depositary, building, and thus a mapping of the annual evolution of SFE protection, as well as the countries that own the protected technology. The methodology used in the research for analysis and discussion of the scientific and technological data is described in Fig. 1.

Research in Scientific Papers Database

A search in the *Science Direct, Wiley Online Library* and *SciELO* (*Scientific Electronic Library Online*) databases was performed to seek the main works published in the SFE area, using a combination of keywords related to the area of interest. The scientific papers that use the SFE applied to food, extraction of bioactive and/or functional compounds from natural matrix, and new applications of these techniques were selected.

Research in Patent Databases

For the research of the technology protected or described in patent documents related to SFE, a search strategy combining the International Patent Classification fields was performed, in which the documents belonging to this technology are classified, associated with a group of keywords (supercritical extraction, supercritical fluids, extraction with supercritical fluids, supercritical carbon dioxide, critical temperature, critical pressure) that represent the different ways this technology could be identified in the documents. Using this method, a search was performed in the online database of the European Office Espacenet (EP), which includes patents deposited and published in over 80 countries, including, for example, patent requests deposited in Brazil (*Instituto Nacional de Propriedade Industrial* – INPI), in America (*United States Patent and Trademark Office – USPTO*) and through the *Patent Cooperation Treaty* (PCT). It is important to highlight that for the sake of search in patent databases there is an access restriction of 18 months prior to its publication. This is characterized by the Intellectual Property World Organization as the secrecy period.

A prospective study was realized through the collection, treatment, and analysis of the information extracted from the patent documents. The term "patent document" includes published patent requests and granted patents. In order to interpret the information about the protected technology about SFE, each document was analyzed and the relevant information describing the invention was extracted, generating graphs showing the countries and firms owners of this technology, as well as the production and application areas. The use of prospective or future studies to assist decision making is a relatively recent activity and originates from a context of deep changes, especially regarding the globalization of the economy and the acceleration of technological advances.

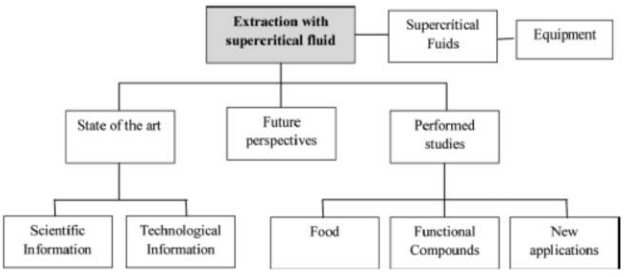

FIG. 1. Methodology applied for the analysis and discussion of scientific and technological data.

PRINCIPLES AND INSTRUMENTATION OF THE SUPERCRITICAL EXTRACTION PROCESS

Supercritical Fluids

The extraction process using supercritical fluids is presently considered a feasible alternative for conventional extraction methods. The solvents in supercritical state show intermediate physical-chemical properties similar to that of liquid and gas, which increases the extracting power of the solvent. The high density of these fluids gives them a high solvation power, whereas its high diffusion and low viscosity values provide a desired penetration power in the solid matrix (37,38).

The high solvation power of supercritical solvents is related to their density. This characteristic promotes higher solubility of the compounds in supercritical fluids when compared with organic solvents. Generally, the solubility of the solutes in supercritical fluids increases with temperature at constant pressure. However, the effect of temperature on pressurized systems is complex due to two factors: an increase in temperature increases the vapor pressure of the solute, promoting an increase of its solubility in supercritical

fluids; on the other hand, an increase in temperature also decreases the solvent density, reducing the solubility of the solute in the solvent. This behavior is known as retrogradation and is found in different systems with compounds from food and natural products (39–44), pharmaceutical (45–47) and aromatic/polyaromatic compounds (48–51); and petroleum fluids (52). The inversion of solubility is easily observed in diagrams as that presented in Fig. 2 (39). Beside the effect of temperature and pressure, the mass molecular and polarity of the solute, the position of a functional group in the molecule, the solid matrix interactions, the location in the matrix also affect the solubility of a solute in supercritical fluids, and the kind of supercritical solvent used (53). Due to the importance of this parameter, several studies addressing this issue have been performed during recent years.

At present, there are various types of solvents being used as supercritical fluids; however, some are found in more reasonably acceptable conditions, but in many cases are not adopted because they require special handling (ammonia, benzene, and cyclohexane). The fluids used in the supercritical extraction should present certain requirements such as: good solubility of the solute to be extracted, be inert to the matrix, and of easy separation from the product, have low cost, and not too high critical pressure. Table 1 shows the critical conditions of various gases and liquids that could be used in the supercritical extraction.

The CO_2 has been widely used as a supercritical fluid in various areas, especially due to the low critical temperature (31.10°C) and low critical pressure (73.76 bar), which has advantages in the conservation of thermolabile substances, easy obtainment of critical conditions (Table 1). The ethane and the ethylene also have reasonable critical properties; however, the cost presents a disadvantage for these solvents.

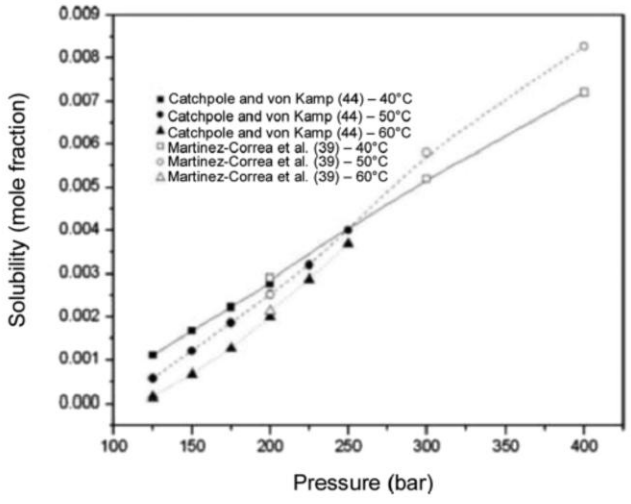

FIG. 2. Modeling of the solubility of squalene in supercritical carbon dioxide. Figure adapted (39).

TABLE 1
Critical properties that can be applied in the supercritical extraction

Solvent	Critical temperature (°C)	Critical pressure (bar)	Reference
Water	374.20	220.48	(54)
Ammonia	132.50	112.77	(55)
Benzene	289.00	48.94	(56)
Ciclohexane	280.30	40.73	(57)
Carbon Dioxide	31.10	73.76	(58)
Ethane	32.30	48.84	(59)
Ethanol	240.75	61.40	(2)
Ethylene	9.30	50.36	(60)
Propane	96.70	42.45	(61)
Propylene	91.90	46.20	(62)
Tolueno	318.60	41.14	(63)

In that sense, the CO_2 becomes the mostly used solvent, especially regarding food or natural products. Besides the CO_2, other supercritical fluids, such as water (54,64) and propane (61,65–67) have been evaluated and presented a high potential for extraction of bioactive compounds. However, the large disadvantages presented by these fluids are the higher critical temperature and the inflammability in the case of propane.

Due to its low polarity, the utilization of CO_2 as a supercritical fluid for the extraction of polar compounds is unviable. However, the addition of a modifying polar solvent or a co-solvent (68,69) can act significantly, altering the characteristics of the process, and thus favoring the extraction of polar compounds. Some works show the optimization of the extraction process through the utilization of a relatively small percentage (1 to 10%) of a co-solvent, widening, and thereby the applications of CO_2 as a fluid for the extraction of polar compounds, such as the utilization of methanol (70), ethanol (71–77), ethane (78), and isopropyl alcohol (17) as modifiers. Although there are many alternatives, ethanol has been the most used modifier in the presence of supercritical CO_2, especially due to its low toxicity, easy availability, and for being considered a GRAS type solvent.

Experimental Models of SFE

The first application in large scale of the supercritical technology in the food industry emerged in Germany for coffee and tea decaffeination, extraction of hop oleoresin, and posterior lower level applications in scents extraction, dyes, and in the cosmetic and pharmaceutical industries. At present countries such as the United States, France, Italy, China, and South Korea rely on supercritical extraction

plants for obtaining pharmaceutical products; India for spices and scents extraction and plants of medium scale in Spain and England apply supercritical chromatography for obtaining nutraceuticals, and the United States operates one of the largest plants in the world for paints manufacturing using oils of vegetable matrix obtained through SFE (79).

The extraction and fractionating of products with supercritical fluids can be realized under two operation models—selective extraction and/or selective separation. The former involves the solvation capacity of the fluid used in the extraction through the manipulation of the operational conditions (temperature and pressure) and/or the modification of the chemical nature of the solvent (addition of a co-solvent). In the latter operation mode, a selective separation is obtained through an adjustment in the depressurization condition, which can be single or serial, allowing a controlled fractionating of the extractable products. The selective separation can also be obtained through the combination of the extraction process to another separation process, such as adsorption (5,13,80).

The choice of experimental method for the study of samples in SFE is conditioned, mainly due to the physical-chemical nature of the substances involved in the process, and to the type and structure of the raw material from where the desired compound will be extracted (13,81). Besides that, process conditions such as temperature, pressure, type of supercritical fluid, solvent outflow, number of extractors, among other factors, exert influence and should be defined for each type of material used.

The operational system used for the extraction with supercritical fluids can be relatively simple or highly complex (15). Basically, it is possible to differentiate two distinct models, the analytical instruments and the preparative systems (in pilot and industrial scales). The analysis systems are used in sample preparation, before, for example, of a chromatographic analysis, with the objective of obtaining milligrams to grams of extracts. The preparative systems are used to extract grams or kilograms of compounds in pilot and industrial scales. In the preparative systems, two different configurations can be found, the processing of solid or liquid samples (6,14).

Generally, a preparative system in a pilot scale plant (Fig. 3) consists of a pump for the solvent, which pumps the liquid in the whole system, a pump for the modifier or co-solvent, if necessary, an extraction cell when the configuration of the system is for solids, or an extraction column when used for liquids. Additionally, one or more separators, also called fractionating cells, are used where the extract is collected and the solvent depressurized.

The extraction cells or columns and the fractioners of the system are generally equipped with independent temperature and pressure controllers, in such a way that the fractionating of the compounds extracted from the matrix can

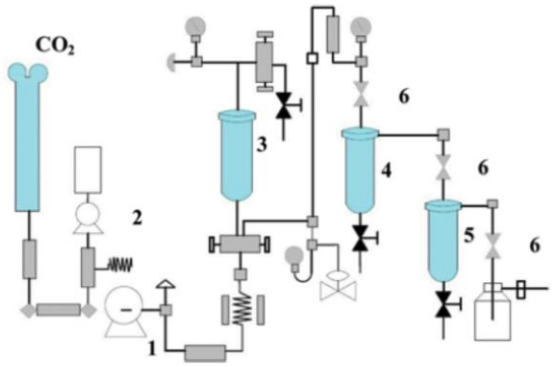

FIG. 3. Project of a pilot extraction plant with supercritical fluid equipped with two fractionating cells. (1) CO_2 pump, (2) co-solvent or modifier pump; (3) extraction cells for solid samples; (4) fractionating cell 1; (5) fractionating cell 2; (6) valves. Figure adapted (14). (Color figure available online)

be performed by a modification mechanism of the operational systems. Separators placed in series in distinct conditions can be applied and therefore different compounds can be separated, depending on the solubility of the compound in the supercritical fluid given at the condition applied in that separator.

Various conformations can be presented in order to obtain a desired compound. The alterations in the operation forms always have the aim to optimize the process, whether it is a semi-continuous extraction (82,83) or in steps (84,85), with the use of serial separators (11,86) or with the use of pressure profiles (87,88). Depending on the objective of the process, various accessories can be added to the system, such as an automatic sampler, and a cooling system especially designed to capture volatile compounds, among others.

The main difference between the pilot plants for the processing of solid or liquid samples is the use of an extraction cell or an extraction column, respectively (89). The processing of solid is always performed in batches and in a non-continuous or semi-continuous process, and generally, the processing of liquids is performed under counter-flow conditions in a continuous way. In the extraction of liquid samples, the supercritical fluid moves in the counter-flow to the sample feeding (14).

The selection of the operation conditions of the SFE process is mainly based on the (solute) solubility in the solvent and/or on the global extraction efficiency, which is defined as the maximum quantity of material that can be extracted at a certain temperature, pressure and outflow. The basic engineering for projects of equipments which use supercritical fluids is available; however, little data has been published regarding the calculation of the increase in scale of supercritical extraction unities (90). According to Meireles (91) the costs involved for the utilization of SFE pose an

obstacle to the dissemination of this technology, since the process potential and the great variety of high quality products that can be obtained are evidenced. However, it is necessary to know the available options, so that the chosen method can be the most adequate.

SFE APPLIED IN THE EXTRACTION OF BIOACTIVE AND/OR FUNCTIONAL COMPOUNDS FROM NATURAL MATRIX

Various vegetable matrixes have been used as sources of bioactive and/or functional compounds. Among the different compounds with functional properties extracted by SFE from natural sources, the antioxidants stand out as the most studied compounds (92,93). These compounds perform an important role in the food (nutraceuticals) and drugs technology due to its utility against the lipid peroxidation.

Despite the various works published in the area of functional compounds extracted from natural matrix using SFE, the most usual methods chosen to obtain these compounds are the conventional ones, such as the liquid-liquid extraction, maceration, and extraction with organic solvent. Such methods are a disadvantage; the fact that they are arduous and time consuming processes, present low efficiency, and low selectivity in the extractive process. Besides that, these traditional techniques employ a great quantity of toxic solvents, which causes the need to use additional fractionating phases for removal of the solvent and undesirable components, thereby increasing the time and cost of the process. On the other hand, the SFE, which is a faster technique, presents a high efficiency and selectivity, and does not produce organic residues; however, the high cost of the equipment still limits its utilization.

One of the main aspects that should be considered in the SFE is the choice of the operational conditions in the extraction process. The use of the optimized values for the different conditions can significantly improve the efficiency and recuperation of a target compound. With the objective of effectively optimizing the conditions that influence the process (temperature, pressure, time, type, and percentage of modifiers or co-solvents, size of the sample particle, among others), different approaches have been applied in the studies involving SFE (5,94–97). Table 2 lists the main and most recent works found in the literature using SFE as a technique for obtaining functional compounds from the vegetable matrix, as well as the conditions of extraction used in the studies.

There are various studies in the literature comparing the antioxidant activity of extracts obtained from plants using conventional extraction methods and the SFE thus showing the benefits presented by this technique. Stashenko et al. (98) evaluated the volatile secondary metabolites obtained from fresh leaves and sticks of rosemary (*Lippia alba*), using various extraction techniques (hydrodistillation, simultaneous distillation solvent extraction) and SFE using supercritical CO_2. The authors isolated over 40 bioactive compounds when using the SFE technique, thereby obtaining more complex extracts, with a higher quantity of sesquiterpenes when compared to the remaining techniques used. Yépez et al. (79) isolated extracts with high antioxidant activity from coriander (*Coriandrum sativum*), using SFE with CO_2. When compared to other extraction techniques, the extracts obtained by SFE presented a higher efficiency and higher antioxidant activity.

Sun et al. (70) extracted the active substance paeonol which presents anti-inflammatory activity from *Cynanchum paniculatum* using SFE and other conventional techniques. The authors managed to optimize the extraction conditions (150 bar, 55°C, 1.5 hours, and 40–60 mesh particles) and obtained a raw extract efficiency of 6.02% containing 72,02% of the active substance paeonol, a very superior result to that found by other extraction techniques (extraction by ultrasound 1.56%, vapor distillation 1.64%, and Soxhlet 2.74%). The *in vitro* antioxidant activity of the lotus germ oil (*Nelumbo nucifera*) extracted by SFE was investigated through the determination of phenolic and tocopherol compounds by Li et al. (99). Twenty-one functional compounds were identified in the oil extracted by SFE, comprising around 92.13% of the total oil, determining a percentage of 9.0% of phenolic compounds. Table 3 shows some works comparing SFE with other extraction techniques, as well as the raw material used and the process efficiency.

In all works evaluated which compared the use of SFE to other conventional extractive methods, it was observed that the quantity of compounds obtained by SFE from the same matrix is very superior. However, despite the higher number of compounds extracted, the efficiency of the extraction process is often lower, which indicates a higher selectivity. On the other hand, when conventional methods were used, a higher yield was obtained when the extraction by Soxhlet is used; however, the extract normally obtained is not selective, that is, undesirable compounds were also extracted together with the target compounds, leading to a higher efficiency.

Among the natural matrix, the scented plants have been widely studied for the obtainment of antioxidant compounds. Studies involving SFE from functional compounds of *Zingiber officinale Roscoe* (ginger) (17,68), *Melissa officinalis L.* (melissa) (100), *Rosmarinus officiniali L.* (rosemary), and *Curcuma longa L.* (saffron) (17) were successfully realized in obtaining extracts with proved antioxidant activity. Zancan et al. (68) evaluated the effects of temperature, pressure, and the addition of a co-solvent on the kinetics of ginger oil-resin extraction. It was observed by the authors that the addition of a co-solvent was not necessary to increase the efficiency of the extracting process. The best antioxidant properties from the extracts were obtained when the extraction was performed in low temperatures

TABLE 2
Main works published about the recuperation of bioactive and/or functional compounds in natural matrix using SFE and the conditions of the process

Natural matrix	Common name	Functional compound	Functional activity	Extraction conditions	Reference
Lippia alba	Rosemary	Volatile Secondary Metabolites	Antioxidant	CO_2, 200 bar, 50°C	(98)
Coriandrum sativum	Coriander	Carotenoids	Antioxidant	CO_2, 117 bar, 45°C	(79)
Cynanchum paniculatum	–	Paeonol	Anti-inflammatory	CO_2 + methanol, 150 bar, 55°C	(70)
Nelumbo nucifera	Lotus	Germen oil	Antioxidant	CO_2, 320 bar and 50°C	(99)
Stevia rebaudiana B.	Stevia	Glycosides	Hypoglycemic Hypotensive	CO_2, 120, 200 and 250 bar, 30°C	(69)
Salvia hispanica L.	Sage	Oil	Hipocohlesterolemic	CO_2, 250, 350 and 450 bar, 40, 60 and 80°C	(100)
Zingiber officinale Roscoe	Ginger	Oleoresin	Antioxidant Antimicrobial	CO_2, 200 bar, 25°C	(68)
Melissa officinalis L.	Melissa	Phenolic Compounds	Antioxidant	CO_2, 100 bar, 35°C	(101)
Rosmarinus officinialis L.	Rosemary	Volatile Oil	Antioxidant Antimicrobial	CO_2, 100 bar, 30°C	(17)
Curcuma longa L.	Saffron	Volatile Oil	Antioxidant Antimicrobial	CO_2 + isopropilic alcohol, 300 bar, 40°C	(17)
Salvia mirzayanii	–	Essential Oil	Antimicrobial	CO_2 + methanol, 355 bar, 35°C	(102)
Chamomilla recutita L.	Chamomile	Oleoresin	Anti-inflammatory Anti-spasmodic	CO_2, 160 and 200 bar, 30 and 40°C	(103)
Salvia hispanica L.	Sage	Oil	Hipocholesterolemic	CO_2, 136, 272 and 408, 40, 60 and 80°C	(104)
Matricaria chamomilla	German Chamomile	Essential Oil	Anti-inflammatory Anti-spasmodic	CO_2, 250 bar and 40°C	(105)
Salvia officinalis L.	Sage	Essential Oil	Anti-spasmodic	CO_2, 128 bar, 50°C	(106)
Nigella sativa	Black cumin	Essential Oil	Antimicrobial	CO_2, 400 bar, 40°C	(107)
Foeniculum vulgare	Anis eed	Triglycerides	Anti-spasmodic Diuretic	CO_2, 300 bar and 40°C	(82)
Hierochloe odorata	Sweet grass	Coumarin	Antioxidant	CO_2, 250 to 350 bar, 40°C	(108)
Eugenia caryophillus	Clove bud	Essential Oil	Antiseptic	CO_2, 90 to 120 bar, 50°C	(109)
Rosmarinus officinialis	Rosemary	Essential Oil	Antioxidant	CO_2, 100 to 400 bar, 40 to 60°C	(110)
Pistachia vera	Pistachio	Extract	Antioxidant	CO_2, 220 bar, 50°C	(111)
Cordia verbenacea	"Erva-baleeira"	β-caryophyllene	Anti-inflammatory	CO_2, 80, 200 to 300 bar, 50 and 60°C	(112)

and pressures (200 bar and 25°C). Leal et al. (17) realized the comparison of antioxidant and antimicrobial activities of three scented plants, ginger (*Zingiber officinale Roscoe*), rosemary (*Rosmarinus officinialis L.*), and saffron (*Curcuma longa L.*) using supercritical CO_2 with and without solvent (isopropyl alcohol). They concluded that the rosemary extracts (100 bar, 30°C) presented better antioxidant activities, whereas the saffron extracts (300 bar, 40°C) presented the better antimicrobial properties after the optimized SFE process in the presense of a co-solvent.

Another example is the extraction of essential oils. Despite the facility of the conventional technique (hydrodistillation),

TABLE 3
Main works published about the comparison in the obtainment of natural matrix compounds using SFE and other conventional extraction techniques

Raw-material (Common name)	Technique	Number of extracted compounds	Extraction (%)	Solvent used	References
Lippia alba (Rosemary)	SFE	44	57,0	Carbon Dioxide	(98)
	Simultaneous Destilation	30	40,0	Dichloromethane	
	Hydrodistillation	30	41,0	Water	
Cynanchum paniculatum (Coriander)	SFE (150 bar, 55°C)	1	4,30	Carbon dioxide and methanol (6%)	(70)
	Soxhlet	1	2,74	Methanol	
	Extraction by Ultrasound	1	1,56	Methanol	
	Vapor Destilation	1	1,64	Water	
Matricaria chamomilla (Chamomile)	SFE (250 bar, 40°C)	3	3,81	Carbon dioxide	(105)
	Soxhlet	2	10,0	Ethanol	
	Maceration	1	8,90	Ethanol	
	Vapor Destilation	2	0,60	Water	
Pogostemon cablin (Pachouli)	SFE (CO_2, 85 and 140 bar, 40 and 50°C)	18	5,07	Carbon dioxide	(113)
	Vapor Destilation	73	1,50	Water	
Eugenia caryophyllata (Clove Bud)	SFE (100 bar and 50°C)	22	16,9	Carbon dioxide	(114)
	Soxhlet	11	41,8	Ethanol	
	Hydrodistillation	12	11,5	Water	
	Vapor destilation	13	10,1	Water	
Marchantia convoluta (Liverwort)	SFE (320 bar and 40–60°C)	11	73,62	Carbon dioxide	(115)
	Soxhlet	10	74,04	Petroleum ether	
Chrysobalanus icaco (Cocoplum)	SFE (200 bar, 50°C)	25	0,77	Carbon dioxide	(116)
	Soxhlet	13	–	Ethanol	
	Hydrodistillation	6	1,00	Water	
Euphorbia macroclada (Spurge Arabic)	SFE (400 bar, 50°C)	19	5,80	Carbon dioxide and methanol (10%)	(117)
	Soxhlet	21	1,10	Methylene chloride	
Alnus glutinosa (Black alder)	SFE (300 bar, 60°C)	8	2,56	Carbon dioxide	(118)
	SFE (300 bar, 60°C)	8	3,81	Carbon dioxide and ethanol (10%)	
	Soxhlet	8	2,30	Hexane	
	Soxhlet	8	40,9	Ethanol	
Salvia officinalis (Sage)	SFE (300 bar, 50°C)	45	4,82	Carbon dioxide	(119)
	Soxhlet	36	26,5	Ethanol/Water (70:30)	
	Hydrodistillation	62	0,50	Water	

the method involves some inconvenient aspects, such as thermal degradation, hydrolysis, and solubilization in water of some compounds which alter the flavor and scent of many essential oils. However, the use of SFE has been a good alternative for obtaining these compounds. Kotnik et al. (105) compared the antioxidant activity of the chamomile essential oil (*Matricaria chamomilla*) obtained by SFE and other techniques, and verified that the higher content of active compounds and the higher efficiency were obtained using the SFE (250 bar and 40°C). When compared to the conventional extraction techniques, sometimes the efficiency of the extractive process obtained by SFE is lower. However, it is observed that the selectivity of the process and the quantitative obtainment of functional interest compounds are often very superior when SFE is used.

Another natural compound of great application in the food, pharmaceutical, and cosmetic areas is propolis, which is a complex resinous mixture produced by bees through the mixture of exudate of different plants and wax and salivary secretions (120–122). Numerous studies have proved its

antioxidant (123–125), antimicrobial (120,126,127), noninflammatory (128), anticancer (76), and anti-HIV (129) activities. These biological activities are attributed to compounds such as phenolic acids, flavonoids, terpenes, and sesquiterpenes (73,120) alcoholic extraction being the most used for obtaining these biocompounds, even though it presents numerous disadvantages in the extractive process when compared to SFE.

The Chinese patent CN1258511 describes the extraction of active compounds from propolis by SFE, demonstrating that the extracts obtained by CO_2 + ethanol are rich in different compounds (polysaccharides, flavones, and terpenes), thus being a highly efficient and selective method for the extracting process (130). Table 4 presents the main works using the SFE as a method for the obtainment of propolis bioactive compounds.

Biscaia and Ferreira (20) compared the efficiency obtained from propolis extraction using different extractive procedures, such as SFE using CO_2 with and without the co-solvent, as well as by conventional methods, Soxhlet and maceration. The SFE method using the ethanol as a co-solvent increased the efficiency of the extraction of bioactive compounds approximately three times in comparison to the extraction using only CO_2.

The anti-cancer activity from propolis extract was evaluated through the obtainment of the active compound Artepillin C (DHCA -3,5-diprenyl-4-hydroxycinnamic acid) present in the matrix using SFE (76). In another study, the antioxidant activity of the Artepillin C (DHCA) serial lipids of low density obtained by SFE was evaluated (74). This study individually investigated organic solvents and supercritical CO_2 to recuperate the Artepillin C (DHCA) from Brazilian propolis. The experimental results indicated that the temperature of the process and the rate of addition of ethyl-acetate, used as co-solvent, are two factors that directly influence the recuperation and purity of the extracts.

SFE APPLIED TO FOOD

Various studies published in the last decade make reference to the utilization of this technique for the obtainment of different compounds from a great variety of food samples, such as essential fruit oils (58,132–134), fatty acids from vegetable oils (seeds), and animals (fish) (135) and bioactive compounds, such as carotenoids, sitosterols, tocopherol, and alkaloids, from fruits and vegetables (15,24,73,96,136,137). Besides that, SFE has been used for the extraction and fractionating of carbohydrates (27,138,139) of fatty acids in fish oils (10,140–144) for obtaining amino-acids profiles in soy and corn transgenic samples (145) and fatty acids profiles in corn transgenic samples (146), among others. In Table 5, the main works published about the compounds obtained from food matrix using SFE are listed, as well as the conditions of the process used.

Regarding vegetable oils, various works have evaluated the influence of the variables used in SFE on the efficiency of extraction, recuperation, and composition of the oils extracted from various food sources (10,148). In the traditional methods for lipids extraction with organic solvents, due to the extraction conditions, the integrity of the compounds extracted and the matrix can be affected by the thermal decomposition of contamination by the solvent. The SFE provides an alternative method to remove lipids, without causing a significant reduction in the organoleptical properties of the product.

The use of co-solvents is much more necessary with the objective to optimize the extraction process of relevant compounds, since the presence of these substances in lower quantities is responsible to increase the efficiency of the process. In the face of the importance of these modifiers, different studies have been realized in the food matrix. Kitzberger et al. (75) evaluated the antioxidant and antimicrobial activity from the extract obtained from mushroom (*Lentinula edodes*) by SFE using CO_2 pure

TABLE 4
Main works published about the recuperation of bioactive compounds of propolis using SFE and process conditions

Functional compound	Functional activity	Extraction conditions	Reference
Phenolic Compounds Flavonoids	Antimicrobial Anti-inflammatory	CO_2, 100–250 bar, 30, 40 and 50°C	(20)
Artepillin C (DHCA)	Anti-cancer	CO_2 + ethanol, 207 bar, 40, 50 and 60°C	(76)
Flavonoids	Antioxidant	CO_2 + ethanol, 276–345 bar, 45°C	(71)
Phenolic Compounds	Antioxidant	CO_2, 200 bar, 60°C	(125)
Artepillin C (DHCA)	Antioxidant	CO_2 + ethanol, 50 bar, 50°C	(74)
Flavonoids	Antimicrobial	CO_2 + ethanol, 300 bar, 60°C	(73)
Artepillin C (DHCA)	Anti-cancer	CO_2 + ethanol, 200 bar, 55°C	(77)
Fenolic Compounds	Antimicrobial Antioxidant	CO_2, 150 and 350 bar, 60°C	(131)

TABLE 5
Main works published about the recuperation of compounds in food matrix using SFE and process conditions

Food matrix	Functional compounds	Extraction conditions	References
Rice flour (*Oryza Sativa*)	Tocotrienols, tocopherols and sterols	CO_2, 100–400 bar, 50 and 60°C	(147)
Hazelnut (*Corylus avellana*)	Oil	CO_2, 300–600 bar, 40 and 60°C	(148)
Cocoa (*Theobroma cacao*)	Triglycerides	CO_2 and CO_2 + ethanol, 200–400 bar, 70°C	(149)
Onion (*Allium caepa*)	Oil	CO_2, 207–287 bar, 37–50°C	(150)
Mushroom (*Lentinula edodes*)	Phenolic Compounds	CO_2 and CO_2 + ethanol, 200–300 bar, 40°C	(75)
Cupuaçu (*Theobroma grandiflorum*)	Fat	CO_2 and ethane, 248–352 bar, 50 and 70°C	(55)
Damask - bagasse (*Armeniaca vulgaris Lam.*)	β-carotene	CO_2, 300 bar, 40°C	(151)
Guarana - seeds (*Paullinia cupana*)	Alkaloids	CO_2 e CO_2 + ethanol, 100–400 bar, 40 to 70°C	(72)
Sunflower - seeds (*Heliantus annuus L.*)	Oil	CO_2, 200–600 bar, 40–80°C	(152)
Orange - peel (*Citrus sinensis*)	Essential Oil	CO_2, 200 bar, 100°C	(153)
Palm oil (*Elaes guineensis*)	Carotenoids	CO_2, 250 and 300 bar, 45 and 55°C	(154)
Fish (*Pelodiscus sinensis*)	fatty acids ω-3	CO_2, 60 bar, 80°C	(141)
Peach - seeds (*Prunus persica*)	Essential oils	CO_2 e CO_2 + ethanol, 150 e 198 bar, 40 e 51°C	(134)
Pepper (*Capsicum sp*)	Triglycerides	CO_2, 220–500 bar, 35–65°C	(155)
Parsley - seeds (*Petrosolium sativum*)	Oil	CO_2, 100 e 150 bar, 35 e 45°C	(156)
Tomato (*Solanum lycopersicum*)	Carotenoids, sitosterols, tocopherol	CO_2, 460 bar, 80°C	(137)
Grapes - bagasse (*Vitis vinifera*)	Phenolic Compounds	CO_2 + ethanol, 150 bar, 40°C	(157)
Grapes - seeds (*Vitis Vinifera*)	α-tocopherol	CO_2, 250 bar, 80°C	(96)
Grape - seeds (*Vitis Vinifera*)	Oil	CO_2, 160, 180 e 200 bar, 100°C	(158)

and with the addition of a co-solvent (ethanol) and by extraction with low pressure. The extracts obtained by SFE (200 to 300 bar and 40°C) using 15% ethanol as a co-solvent presented the best antioxidant activities, when compared to other substances as co-solvent evaluated methods (cold maceration with n-hexane, ethyl acetate, and dichloromethane).

Different studies report the use of a co-solvent for the obtainment of carotenoids from food matrix (149,151,159,160) in the SFE process using supercritical CO_2. In the study realized by Vasapollo et al. (159) an innovative process was developed for the extraction of lycopene from tomato using supercritical CO_2 (335–450 bar, 45–70°C) in the presence of vegetable oil as a co-solvent (hazelnut oil). The presence of the co-solvent improves the efficiency of the lycopene extract obtained and has a beneficial role in the stability of the pigment. Ethanol (2–28%) was used as co-solvent to optimize the process of obtaining β-carotene from the damask bagasse, using CO_2 as supercritical fluid, temperature of 43 to 77°C, and pressure 133–473 bar (160).

The use of co-solvents has also been applied in SFE to remove compounds from certain matrix, for example, the removal of caffeine from coffee (161) and from green tea (162,163), from bitter compounds of beer hop (67), among others.

The SFE technique using CO_2 as supercritical fluid and water as co-solvent was applied to selectively extract caffeine from green tea, avoiding the extraction of antioxidants, compounds considered desirable from the matrix (162). The selectivity of the extraction conditions studied in the process was confirmed by HPLC, demonstrating that the presence of water as a co-solvent allows the optimization of caffeine removal, without altering the other compounds present in the sample. However, in the study realized by Sun et al. (163), the water did not turn out to be an adequate co-solvent in the process of removing caffeine from green tea by SFE, the use of ethanol, in that case, being the most indicated as a co-solvent.

Utilization of Agroindustrial Residues Using SFE

Another area of application of SFE in food is in utilization of residues, since a wide variety of sub-products is generated from the industrial activities of the food industry. In view of this, various studies in this area (164) resulted in products with high added value. Grape seeds are classified as residues of the wine industry and have a valuable utilization when treated with the organic solvent *n*-hexane in order to obtain the oil from this seed. The grape seed oil is considered a subproduct with excellent nutritional properties due to the high content of unsaturated fatty acids. Bravi et al. (96) extracted α-tocopherol from grape seeds (*Vitis Vinifera*) with supercritical CO_2. The results obtained for SFE (250 bar, 80°C) were compared to the extraction with *n*-hexane, where the concentration of α-tocopherol in the oil extracted with supercritical CO_2 was very superior to that obtained by the extraction with *n*-hexane; however, the general efficiency of the oil was lower for SFE.

The α-tocopherol was extracted from olive leaves (residue from olive harvest) by De-Lucas et al. (165). Extracts highly concentrated, containing 74.5% and 97.1% from α -tocopherol were obtained by SFE (250–450 bar, 40–60°C), and these concentrations were very superior to those obtained by Soxhlet. Danielski et al. (147) used rice flour (sub-product of the grains polishing) and obtained an extract rich in active compounds using SFE with different pressure and temperature conditions (100–400 bar, 50 and 60°C). Doker et al. (151) evaluated the obtainment of β-carotene from damask bagasse (residue of the juice industry), using supercritical CO_2 as solvent. The values optimized for pressure and temperature were 300 bar and 40°C, respectively, with a particle size of 106 μm.

Machmudh et al. (166) evaluated the lycopene extraction from tomato peel, and the obtainment of oil from the seeds, using supercritical CO_2. The authors reported that the presence of the seeds oil helped to improve the recuperation of lycopene from 18% to 56%, having therefore a beneficial role in the stability of carotenoid extraction, due to its fatty acids content.

SFE Applied to Genetically Modified Food Samples

Recently, SFE has been applied in the food area for the determination of amino-acids profiles in different genetically modified soy and corn varieties. The extraction occurs with the presence of a co-solvent, methanol (35%), and the identification of amino-acids is realized by GC-MS (*Gas Chromatography–Mass Spectrometry*). The extraction with CO_2 allowed a faster and more efficient recuperation of amino-acids from soy and corn grains, in comparison to other previously used techniques (145). Toribio et al. (146) evaluated different parameters on the extraction of supercritical CO_2 with methanol, for the serial obtainment of neutral and polar lipids from transgenic corn. The best extraction results were reached when 15% (v/v) of methanol and 90 minutes of extraction at 300 bar and 60°C were used. The method developed was successfully applied to obtain the profile of fatty acids from various genetically modified corn samples and its isogenic lines after analysis by GC-MS. Therefore, various genetically modified organisms can be directly compared to its respective non-transgenic varieties through the utilization of SFE as an analytical extraction technique, followed by the identification by GC-MS.

New Applications of SFE: Future Perspectives

In view of the scientific and technological advances presented by SFE in the last years, new application areas have been the object of research involving this technology, such as the pharmaceutical area, chemical residues, bio-fuel, polymer, and other areas. The results of the published research related to SFE contribute to the various advantages and possibilities that the technique offers. With the dissemination of the present knowledge available about the subject, it is possible to easily make the choice of the most adequate raw material, such as the type of supercritical fluid to be used, as well as the parameters of temperature and pressure of the process.

Pharmaceutical Area

At present, the SFE has been applied for obtaining compounds in seaweed and micro-seaweed (14,167,168). These organisms have stood out as raw material for obtaining new sources of functional and natural antioxidant compounds, which enables them to be applied in the food and pharmaceutical industries. There is a great variety of seaweed and micro-seaweed species, with differentiated chemical composition characteristics among them, thereby increasing the interest in finding new sources of bioactive compounds.

Due to the advantages of using SFE, the pharmaceutical industries have been developing new processes with low environmental impact for drugs production, with the objective of reducing the volatile organic compounds in the medicament manufacturing and to avoid the presence of residues in the final product. The new utilization of SFE in the pharmaceutical area includes enantiomeric separations, production of enzymatic reactions in supercritical medium, production of liposomes, and purification of pharmaceutical excipients (169,170).

Chemical Residues (Metal Recuperation)

SFE has been studied in order to be used in the removal of heavy metals from solid (soil, mud, wood, paper, tissue) and liquid (water) matrix, since no other available technique can realize such procedure with efficiency, therefore being considered as one of the most promising techniques

at this time (30,31). Albarelli et al. (31) used the supercritical CO_2 for the removal of heavy metals, such as copper in banana skin and for obtaining antioxidant compounds present in the matrix. The complexing agents used in the process of conventional solvent extraction can also be used in SFE, given that the metallic ions are soluble in supercritical CO_2. According to Sunarso and Ismadji (171), the solubility of metallic complex compounds in supercritical CO_2 can significantly vary depending on the chemical nature of the complex compounds present in the process. Various complexing agents have been employed in the extraction of heavy metals from soil, water, and food samples, using SFE, such as diisooctyl-thiophospinic acid (Cyanex 302), sodium diethylthiocarbamate (Aliquat 336), bis(2-ethylhexyl)phosphoric acid, and bis(2-ethylhexyl) monothiophosphoric acid (32).

Biofuel

A new and important application of SFE is the production of biofuel, and recent works have been published in this new research line (34,35,172–177). This new methodology has, as objectives, the substitution of methanol by ethanol in the transesterification process for obtaining biodiesel from vegetable oils and also the utilization of supercritical CO_2 for the extraction of seeds oil, making the process totally renewable. Gui et al. (178) found the ideal conditions for obtaining biodiesel using SFE.

In the study, palm oil was used as raw material, with the temperature of the reaction process of 349°C, reaction period of 30 minutes, obtaining a final yield of 79.2% of biodiesel. In the study realized by Breet et al. (174), the seeds oil of "pinhão-manso" (*Jatropha curcas*) using supercritical CO_2 was extracted and converted in biodiesel. This work was realized as a scientific support for a world-wide research that had the objective to identify a source of sustainable power produced by clean technology that would come to rapidly substitute the decrease in the use of fossil fuel resources.

The use of supercritical CO_2 is also favorable for the production of bioethanol. Melo et al. (36) developed a process that allowed the availability of glucose from cellulose for obtaining bioethanol through fermentation, after the treatment of sugarcane bagasse with supercritical CO_2 and ethanol as a co-solvent (pressure of 150 bar and temperature of 75°C). Vezzú et al. (179) investigated the possibility of bioethanol production through glucose fermentation using supercritical CO_2 in pressures of up to 48 bar. The fermentation was realized using the yeast strain *Saccharomyces cerevisiae*, which is commonly applied in the production of industrial bioethanol. It was observed from the study that the fermentation under supercritical CO_2 pressure can be a real potential for the recuperation of high purity bioethanol, thereby avoiding the energetically expensive distillation process.

Polymers

The use of supercritical fluids has been the target of research for application in polymers, especially with CO_2 as a solvent in the polymer modification processes, formation of composites, production of microcellular foam particles, and polymerization (180). The polymers processed are used in various industrial applications. Its particles, for example, are used for the manufacturing of paints and drugs and also applied in separation methods that use porous membranes. In the traditional polymer processing methods, which are environmentally dangerous, volatile organic solvents and chlorofluorocarbons are used (8).

Supercritical CO_2 is considered a good solvent for the processing of many polymers with low polarity and compounds that present a low molecular weight. However, it does not present a good efficiency when used in the processing of polymers with high molecular weight (181). Therefore, the solubility of supercritical CO_2 in many polymers is substantial. Its solubility depends upon the temperature and pressure used, and also on the weak interactions with the groups of the polymeric chain. The dissolved CO_2 is responsible for causing considerable reduction in the viscosity of the melted polymer, mainly due to an increase in the free volume. Besides that, it alters the physical properties of the polymers, such as density, diffusivity, and volume. Therefore, it has a great potential as a plasticizer in polymer processing, which is generally realized at high temperatures (8,182).

The research involving the utilization of SFE in the polymer area has been realized by chemists and chemical engineers who search for cleaner methods for the processing of these materials, given its importance in various applications, as an alternative for the decrease in the emission of toxic solvents used in the chemical industry.

Presently, the supercritical CO_2 is also applied in the processing of biodegradable polymers (183) and for pharmaceutical applications in the form of particles and microcellular foam (184). The low thermal stability of biodegradable polymer, together with the lack of adequate organic solvents for the processing of these biomaterials are the main reasons for the use of supercritical CO_2 as a solvent of these new and promising biocomposites.

Combination of Supercritical Fluid Extraction with Other Sample Preparation Methods

Nowadays, analysis techniques using green chemical parameters—clean and sustainable technologies—have received more importance in the scientific and industrial fields, considering the concerns with the environment.

With the development of SFE, the environmental impact issue regarding the compounds extraction has had greater visibility, with the application of this technique (171). This is especially due to the non-use of toxic solvents, the efficiency of the extraction process in critical temperature

and pressure, and the safety and its effects in the samples pre-concentration. Therefore, it is in the interest of researchers to determine and identify better results for extraction and preparation analytical processes. The use of sustainable techniques such as SFE, combined with other compound preparation and extraction techniques, should be ensured, aimed at obtaining more efficient and technologically clean methods.

Among the other techniques studied, combined with SFE, the following ones stand out: Dispersive Liquid-Liquid Microextraction (DLLME) (185–186), a Supercritical Fluid Extraction on-line Headspace Solid-phase Microextraction combined with Gas Chromatography-Mas Spectrometry (SFE in situ derivatization on-line HS-SPME-GC-MS) (187), a Solid-phase/supercritical-fluid extraction (SPE/SFE) (188), a supercritical carbon dioxide and ionic liquid (SFE/IL) (189) and Ultrasound followed by supercritical CO_2 extraction (ULS/SFE) (190).

Certain studies have used the DLLME combined with SFE to determine aromatic polycyclical hydrocarbonates (APH) in marine sediments (185). In the study, it was identified that the technique used for the analysis of APH in different solid samples had great potential. This is due to the fact that the association of SFE-DLLME allows the pre-concentration of APH in the samples, needed before the final analysis. The SFE together with the DLLME, followed by gas chromatography-flame ionization detection (GC-FID) was also applied for extraction and determination of ultra-trace amounts of seven organophosphorus pesticides (OPPs) in soils and in marine sediment samples (186). The combined method applied was considered very efficient for the analysis of concentration determination of OPPs in real soil and in marine sediments samples, considering that the extraction recovery for the target analytes was in the range of 44.4% to 95.4%.

In the study performed by Yang et al. (187), the SFE in situ derivatization on-line HS-SPME-GC-MS was used for the determination of parabens and polyphenolic antioxidants in cosmetics. The preservatives and antioxidants were extracted from the cosmetics matrix with CO_2. The results found by the authors show that the technique used, combined with SFE, were more efficient for the tracking of preservatives and analysis of antoxidants in cosmetics, when compared to the isolated use of SFE or Polyacrylate Solid-Phase Microextraction (SPME).

The deterpenation of two different cold-pressed mandarin peel oils employing countercurrent extraction and ad-/desorption with supercritical CO_2 and a combination of these processes were investigated in the study performed by Danielski et al. (191). The optimal process conditions were 40°C and 80 bar for terpene desorption followed by 200 bar for the desorption of oxygenated components when the combined techniques were used. This shows an increase in the analytical sensitivity of the studied process.

Klejdus et al. (188) describe a new extraction technique based on the combination of solid-phase/supercritical fluid extraction (SPE/SFE) with subsequent reversed-phase HPLC. Different phenolic compounds of freshwater microalgae (*Spongiochloris spongiosa*) and selected cyanobacterial species (*Spirulina platensis, Anabaena doliolum, Nostoc* sp., and *Cylindrospermum* sp.), were extracted and identified, such as benzoic acid derivatives (p-hydroxybenzoic, protocatechuic, gallic, vanillic and syringic acid), hydroxybenzaldehydes (4-hydroxybenzaldehyde and 3,4-dihydroxybenzaldehyde), and cinnamic acid derivatives (o-coumaric, p-coumaric, caffeic, ferulic, sinapic, and chlorogenic acid).

Some recently published studies have reported the use of a combination of supercritical carbon dioxide and ionic liquid in a novel assembly of carvacrol, an antimicrobial reagent (189), and the extraction of sage diterpenes (*Salvia officinalis* L.) using the Ultrasound followed by supercritical CO_2 extraction (190). Additionally, the SFE has been applied combined with the high power ultrasound to perform the inactivation of *Saccharomyces cerevisiae* (192) and *Escherichia coli* (193).

It should be noted, therefore, that research using a combination and/or association of SFE with other techniques report an improvement in the efficiency of the extraction/analysis process of relevant compounds (188–193). This greatly reduces the time spent in the process, showing a higher analytical sensitivity and being environmentally friendly.

Prospective Study about the SFE Through the Research in Patent Documents

Researching the European Office (*Espacenet*) patent database resulted in a universe of 2.314 registers of patent documents referring to SFE. It is important to highlight that this number does not represent the total number of inventions protected in this area, since one patent can be deposited in different countries with the objective of guaranteeing the right of exclusivity to its inventors in markets considered most relevant, once the patent right is territorial.

The extraction of supercritical fluids had its industrial beginning in Germany, at the end of the 1970s, with the removal of the caffeine from coffee (194–196). The first patent deposited relative to the studied technology (SFE) was in 1974 (GB1481958) of British ownership, which describes the technology of a process that involves the supercritical extraction and catalytic hydrogenation of coal derived compounds.

Figure 4 shows the annual evolution of patent deposits related to SFE between 1974 and 2013. From 1974 through 1988, the deposits of requests related to the basic technology about the SFE process applied mainly to the food and pharmaceutical industry. After this period, the

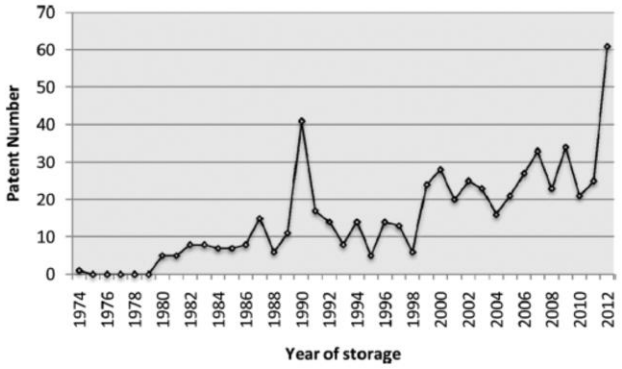

FIG. 4. Annual evolution of patent deposit on SFE between 1974 and 2013.

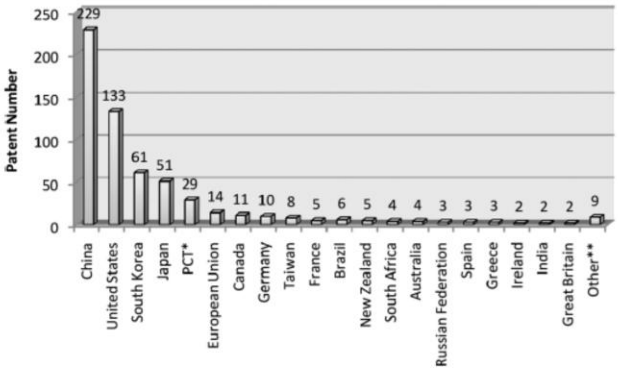

FIG. 5. Distribution of patents deposits about SFE by country of origin of the depositants between 1974 and 2013. *PCT: *Patent Cooperation Treaty*; **Mexico, Hong Kong, Portugal, Poland, Romania, Singapore, Thailand, Malaysia and Hungary: 1 patent by country.

patents related to the SFE applications started to appear, standing out the utilization of this technique for obtaining natural compounds in the area of agriculture, in the determination of pesticides and trace compounds in the food area, and the improvement of the technical processes in the pharmaceutical and cosmetic industries.

The higher number of patent documents deposited about SFE is found in 1990, and that is mainly due to the global policies that encouraged the decrease in the utilization of organic solvents in the different industrial areas, such as the Montreal Protocol. This protocol was introduced in 1987, the main objective being to restrict or eliminate the production and utilization of solvents that interfere in the ozone layer, thereby causing a great deal of interest in the scientific community and the industrial sector in the development of clean technologies, such as the SFE. In 2012, a total of 61 patent registrations were submitted (Fig. 4), showing an increase in interest for the development of new technologies involving supercritical fluids. It is also highlighted that this figure does not represent the real number of patent submissions for the period, since according to the patent legislation, a submission is only available for public consultation after the secrecy period of 18 months. Such a fact explains the lack of patent submissions in 2013.

The analysis of the patents deposited regarding the countries where the protected technology originated—research realized through the identification of the country of origin of the depositary—shows that this technology is very centralized in the more developed countries. Figure 5 relates the number of patent documents deposited in the European office by country of origin, that is, the country of origin of the patent depositary, which is not in secrecy until the moment of the research.

China is responsible for the higher number of patent deposits in the area of methods, extractive processes, and applications of SFE, with 229 documents, followed by the United States, with 133 deposited documents. Based on the surveying of patent documents, it stands out that until 1990 the United States occupied the first position in the ranking of countries that had the higher number of patents related to SFE, and China occupied the fourth position. However, from the 1990s, China moved to the first position in the ranking, mainly due to the investments in education in the country, including in the public policies, the importance of the Research, Development, and Innovation system (RD&I), for economic and social strengthening, and development of the country. Brazil has only six patents deposited in the SFE area, three of which have as owner the *Universidade Estadual de Campinas* (UNICAMP), one in partnership with the *Centro de Tecnologia Canavieira* (CTC), and another with the *Aché Laboratórios Farmacêuticos S.A.*

From the total number of deposited documents, over half of the requests were made by the industrial sector (companies), represented by 62% of these requests, followed by independent inventors constituting 20%. From the deposited patents, only 18% are owned by universities. With this data it is possible to observe that the higher investors in RD&I are in the industrial sector, since it has the higher number of deposited patents.

Regarding the application of the documents, the total volume of deposited patents related to SFE, 34% is related to the food and agrarian sectors, 23% related to the chemical and petro-chemical industries, 17% related to the chromatographic analysis, 11% for the production of drugs and cosmetics, 7% for pesticide analysis, and 3% for contaminants analysis (Fig. 6).

The research applied to SFE in what concerns patents deposit shows the food and agricultural sector as the main application and development field for this technique. Among the documents identified in this area, 39% relate to the extraction of bioactive substances from different natural matrix, 38% for the determination and extraction of oils and fats, and 17% for the determination of residues in food by SFE (Fig. 7).

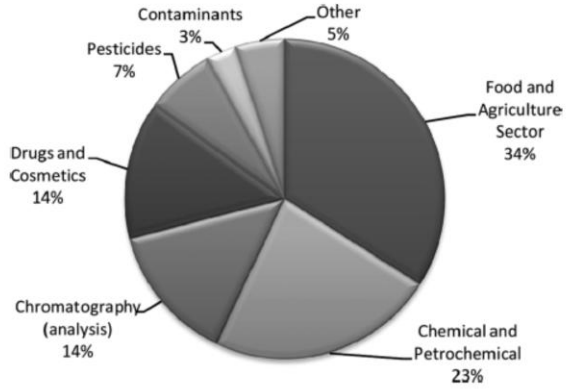

FIG. 6. Distribution of SFE patents deposited by application area between 1974 and 2013.

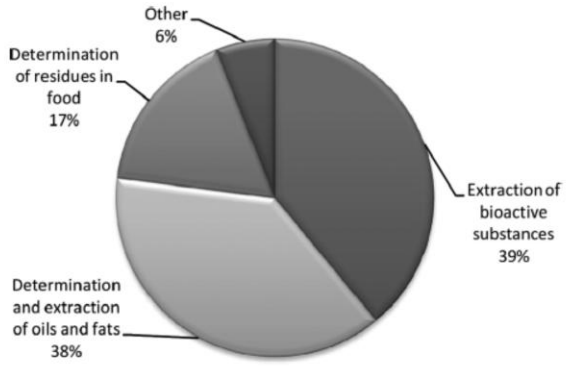

FIG. 7. Distribution of SFE patents in the area of Food and Agriculture between 1974 and 2013.

The number of potential applications for SFE continues to grow globally, which is verified through the increase in patents deposited in the last few years. It is observed that its application is already part of the present scenery, being mainly impelled by the growing demand of high quality products demand and economy's globalization. Besides that, it also stands out in its use in the commerce of pharmaceutical, food, chemical, and cosmetic materials. The increase in the application of this technology in the industrial area is mainly due to the selectivity, facility, and separation capacity that the technique allows in obtaining a great number of organic compounds, of which many are impossible or nonviable to extract through traditional processes, or those whose purification needs high resolution columns, not always available in the national market, thereby making the utilization very costly.

CONCLUSIONS

The use of SFE has been investigated and applied to the food, pharmaceutical, cosmetic, chemical, and biofuel industries of chemical processing, with the possibility of having further developments in this processes due to the growing research in the area. It is presented as a technique that has advantages in the environmental field, since with its utilization there is a reduction in the emission of organic solvent residues, allowing for the utilization of agro-industrial residues through the recuperation of functional compounds, and also the quantification and removal of toxic compounds, such as heavy metals and pesticides in environmental samples.

Presently, the SFE is defined by the scientific community as an alternative technique that should be applied for the obtainment and product development, especially those used for human consumption, such as new functional food, additives, and ingredients for food and pharmaceutical products. With the results of this work it is possible to note that new applications have only appeared in the last few years and the studies about the SFE applications is increasing as shown by the number of new patents and patent deposits.

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REFERENCES

1. Brunner, G. (2000) Fractionation of fats with supercritical carbon dioxide. *Eur. J. Lipid Sci. Technol.*, 102 (3): 240–244.
2. Budich, M.; Brunner, G. (2003) Supercritical fluid extraction of ethanol from aqueous solutions. *J. Supercrit. Fluids*, 25 (1): 45–55.
3. King, M.B.; Boot, T.R. (1993) *Extraction of Natural Products Using Near-Critical Solvents*, 1st ed.; Chapman and Hall: London, UK.
4. Bundschuh, G. (1986) High pressure extraction in the food technology. *ZFL*, 8: 525–534.
5. Brunner, G. (1994) *Gas Extraction: An Introduction to Fundamentals of Supercritical Fluids and the Application to Separation Process*, 1st ed.; Steikopff Darmastadt: New York.
6. Herrero, M.; Mendiola, J.A.; Cifuentes, A.; Ibáñes, E. (2010) Supercritical fluid extraction: Recent advances and applications. *J. Chromatogr.*, 1217 (16): 2495–2511.
7. Chiu, K.L.; Cheng, Y.C.; Chen, J.H.; Chang, C.J.; Yang, P.W. (2002) Supercritical fluids extraction of *Ginkgo ginkgolides* and flavonoids. *J. Supercrit. Fluids*, 24 (1): 77–87.
8. Nalawade, S.P.; Picchioni, F.; Janssen, L.P.B.M. (2006) Supercritical carbon dioxide as a green solvent for processing polymer melts: Processing aspects and applications. *Progress Polym. Sci.*, 31 (1): 19–43.
9. Wang, L.; Yang, B.; Dua, X.; Yi, C. (2008) Optimization of supercritical fluid extraction of flavonoids from *Pueraria lobata*. *Food Chem.*, 108 (2): 737–741.
10. Temelli, F. (2009) Perspectives on supercritical fluid processing of fats and oils. *J. Supercrit. Fluids*, 47 (3): 583–590.
11. Gomes, P.B.; Mata, V.G.; Rodrigues, A.E. (2007) Production of rose geranium oil using supercritical fluid extraction. *J. Supercrit. Fluids*, 41 (1): 50–60.
12. Pourmortazavi, S.M.; Hajimirsadeghi, S.S. (2007) Supercritical fluid extraction in plant essential and volatile oil analysis. *J. Chromatogr.*, 1162 (1–2): 2–24.

13. Brunner, G. (2005) Supercritical fluids: Technology and application to food processing. *J. Food Eng.*, 67 (1–2): 21–33.
14. Herrero, M.; Cifuentes, A.; Ibáñes, E. (2006) Sub- and supercritical fluid extraction of functional ingredients from different natural sources: Plants, food-by-products, algae and microalgae. *Food Chem.*, 98 (1): 136–148.
15. Mattea, F.; Martin, A.; Cocero, M.J. (2009) Carotenoid processing with supercritical fluids. *J. Food Eng.*, 93 (3): 255–265.
16. Mendiola, J.A.; Herrero, M.; Cifuentes, A.; Ibáñes, E. (2007) Use of compressed fluids for sample preparation: Food applications. *J. Chromatogr.*, 8 (1152): 234–46.
17. Leal, P.F.; Braga, M.E.M.; Sato, D.N.; Carvalho, J.E.; Marques, M.O.M.; Meireles, M.A.A. (2003) Functional properties of spice extracts obtained via supercritical fluid extraction. *J. Agric. Food Chem.*, 51 (9): 2520–2525.
18. Pereira, C.G.; Meireles, M.A.A. (2007) Economic analysis of rosemary, fennel and anise essential oils obtained by supercritical fluid extraction. *Flavor Fragrance J.*, 22 (5): 407–413.
19. Safaralie, A.; Fatemi, S.; Salimi, A. (2010) Experimental design on supercritical extraction of essential oil from valerian roots and study of optimal conditions. *Food Bioproducts Processing*, 88 (2): 312–318.
20. Biscaia, D.; Ferreira, S.R.S. (2009) Propolis extracts obtained by low pressure methods and supercritical fluid extraction. *J. Supercrit. Fluids*, 51 (1): 17–23.
21. Zhang, S.; Xi, J.; Wang, C. (2005) High hydrostatic pressure extraction of flavonoids from propolis. *J. Chem. Technol. Biotechnol.*, 80 (1): 50–54.
22. Topal, U.; Sasaki, M.; Goto, M.; Hayakawa, K. (2006) Extraction of lycopene from tomato skin with supercritical carbon dioxide: Effect of operating conditions and solubility analysis. *J. Agric. Food Chem.*, 54 (15): 5604–5610.
23. Huang, W.; Li, Z.; Niu, H.; Li, D.; Zhang, J. (2008) Optimization of operating parameters for supercritical carbon dioxide extraction of lycopene by response surface methodology. *J. Food Eng.*, 89 (3): 298–302, 2008.
24. La-Fuente, J.C.; Oyarz, B.; Quezada, N.; Del-Valle, J.M. (2006) Solubility of carotenoid pigments (lycopene and astaxanthin) in supercritical carbon dioxide. *Fluid Phase Equilib.*, 247 (1): 90–95, 2006.
25. Gelmez, N.; Kincal, N.S.; Yener, M.E. (2009) Optimization of supercritical carbon dioxide extraction of antioxidants from roasted wheat germ based on yield, total phenolic and tocopherol contents, and antioxidant activities of the extracts. *J. Supercrit. Fluids*, 48 (3): 217–224.
26. Meei, M.G.; Keat, T.L., Bhatia, S. (2009) Supercritical carbon dioxide extraction of xanthones with antioxidant activity from *Garcinia mangostana*: Characterization by HPLC/LC–ESI-MS. *J. Supercrit. Fluids*, 49 (3): 330–337a.
27. Montañés, F.; Corzo, N.; Olano, A.; Reglero, G.; Ibáñez, E.; Fornari, T. (2008) Selective fractionation of carbohydrate complex mixtures by supercritical extraction with CO_2 and different co-solvents. *J. Supercrit. Fluids*, 45 (2): 189–194.
28. Tomasko, D.L.; Li, H.; Liu, D.; Han, X.; Wingert, M.J.; Lee, L.J. (2003) A review of CO_2 applications in the processing of polymers. *Ind. Eng. Chem. Res.*, 42 (25): 6431–6456.
29. Sameer, P.N.; Picchiono, F.; Janssen, L.P.B.M. (2006) Supercritical carbon dioxide as a green solvent for processing polymer melts: Processing aspects and applications. *Prog. Polym. Sci.*, 31 (1): 19–43.
30. Wang, W.; Yang, H.J.; Hu, J.; Guo, C.Y. (2009) Extraction of metal ions with non-fluorous bipyridine derivatives as chelating ligands in supercritical carbon dioxide. *J. Supercrit. Fluids*, 51 (2): 181–187.
31. Albarelli, J.; Rabelo, R.B. Santos, D.T.; Beppu, M.M. Meireles, M.A.A. (2011) Effects of supercritical carbon dioxide on waste banana peels for heavy metal removal. *J. Supercrit. Fluids*, 58 (3): 343–351.
32. Nejad, S.J.; Abolghasemi, H.; Moosavian, M.A.; Maragheh, M.G. (2011) Fractional factorial design for the optimization of supercritical carbon dioxide extraction of La^{3+}, Ce^{3+} and Sm^{3+} ions from a solid matrix using bis (2,4,4-trimethylpentyl) dithiophosphinic acid + tributylphosphate. *Chem. Eng. Res. Des.*, 89 (6): 827–835.
33. Meei, M.G.; Keat, T.L., Bhatia, S. (2009) Supercritical ethanol technology for the production of biodiesel: Process optimization studies. *J. Supercrit. Fluids*, 49 (2): 286–292b.
34. Wen, D.; Jiang, H.; Zhang, K. (2009) Supercritical fluids technology for clean biofuel production. *Prog. Nat. Sci.*, 19 (3): 273–284.
35. Paulo, C.I.; Diaz, M.S.; Bringnole, E.A. (2009) Energy Consumption Minimization in Bioethanol Dehydration with Supercritical Fluids. *Comput. Aided Chem. Eng.*, 27: 1833–1838.
36. Melo, S.S.; Rodrigues, F.S.; Kamimura, E.S.; Lacerda, R.S.; Gomide, C.A.; Oliveira, A.L. Availability of cellulose pulp from cane sugar by treatment with sub and supercritical CO_2. *9º Latin American Symposium on Food Science*, Unicamp, Campinas, 2011.
37. Lameira, C.P.; Coelho, G.L.V.; Mothe, C.G. (1997) Extração de lipídeos da amêndoa de castanha de caju com CO_2 supercrítico. *Sci. Food Technol.*, 17 (4): 405–407.
38. Akinlua, A.; Torto, N.; Ajayi, T.R. (2008) Supercritical fluid extraction of aliphatic hydrocarbons from Niger Delta sedimentary rock. *J. Supercrit. Fluids*, 45 (1): 57–63.
39. Martinez-Correa, H.A.; Gomes, D.C.A.; Kanehisa, S.L.; Cabral, F.A. (2010) Measurements and thermodynamic modeling of the solubility of squalene in supercritical carbon dioxide. *J. Food Eng.*, 96: 43–50.
40. Castro-Vargas, H.I.; Benelli, P.; Ferreira, S.R.S.; Parada-Alfonso, F. (2013) Supercritical fluid extracts from tamarillo (*Solanum betaceum Sendtn*) epicarp and its application as protectors against lipid oxidation of cooked beef meat. *J. Supercrit. Fluids*, 76: 17–23.
41. Pereira, C.G.; Rosa, P.T.V.; Meireles, M.A.A. (2007) Extraction and isolation of indole alkaloids from *Tabernaemontana catharinensis*: Technical and economical analysis. *J. Supercrit. Fluids*, 40: 232–238.
42. Rodrigues, R.F.; Tashima, A.K.; Pereira, R.M.S.; Mohamed, R.S.; Cabral, F.A. (2008) Coumarin solubility and extraction from emburana (*Torresea cearensis*) seeds with supercritical carbon dioxide. *J. Supercrit. Fluids*, 43: 375–382.
43. Saldaña, M.D.A.; Mohamed, R.S.M.; Baer, G.; Mazzafera, P. (1999) Extraction of purine alkaloids from mate (*Ilex paraguariensis*) using supercritical CO_2. *J. Agric. Food Chem.*, 47: 3804–3808.
44. Catchpole, O.J.; von Kamp, J.C. (1997) Phase equilibrium for the extraction of squalene from shark liver oil using supercritical carbon dioxide. *Ind. Eng. Chem. Res.*, 36: 3762–8.
45. Shojaee, S.A.; Rajaei, H.; Hezave, A.Z.; Lashkarbolooki, M.; Esmaeilzadeh, F. (2013) Experimental measurement and correlation for solubility of piroxicam (a Non-Steroidal Anti-Inflammatory Drugs (NSAIDs)) supercritical carbon dioxide. *J. Supercrit. Fluids*, in press.
46. Su, C.S.; Chen, Y.P. (2007) Correlation for the solubilities of pharmaceutical compounds in supercritical carbon dioxide. *Fluid Phase Equilibr.*, 254: 167–173.
47. Ko, M.; Shah, V.; Bienkowski, P.R.; Cochran, H.D. (1991) Solubility of the antibiotic penicillin V in supercritical CO_2. *J. Supercrit. Fluids*, 4: 32–39.
48. Sane, A.; Taylor, S.; Sun, Y.P.; Thies, M.C. (2004) A semicontinuous flow apparatus for measuring the solubility of opaque solids in supercritical solutions. *J. Supercrit. Fluids*, 28: 277–285.
49. Yu, E.; Richter, M.; Chen, P.; Wang, X.; Zhang, Z.; Tavlarides, L.L. (1995) Solubilities of polychlorinated biphenyls in supercritical carbon dioxide. *Ind. Eng. Chem. Res.*, 34: 340–346.
50. Shena, B.; Feng, L.; Li, S.; Li, H. (2013) Correlating and predicting the solubilities of polycyclic aromatic hydrocarbons in supercritical fluids using the compressed gas model and the reference solubilities. *Fluid Phase Equilibr.*, 337: 100–108.

51. Spiliotis, N.; Magoulas, K.; Tassios, D. (1994) Prediction of the solubility of aromatic hydrocarbons in supercritical CO_2 with EoS/G E models. *Fluid Phase Equilibr.*, 102: 121–141.
52. Karakas, G.; Dogu, T.; Somer, T.G. (1997) Reactivity of CO_2 during thermal cracking of heavy paraffins under supercritical conditions. *Ind. Eng. Chem. Res.*, 36: 4445–4451.
53. Pereira, C.G.; Meireles, M.A.A. (2010) Supercritical fluid extraction of bioactive compounds: Fundamentals, applications and economic perspectives. *Food Bioprocess Tech.*, 3 (3): 340–372.
54. Anekpankul, T.; Goto, M.; Sasaki, M.; Pavasant, P.; Shotipruk, A. (2007) Extraction of anticancer damnacanthal from roots of *Morinda citrifolia* by subcritical water. *Sep. Purif. Technol.*, 55 (3): 343–349.
55. Bludworth, J.; Knopf, F.C. (1993) Reactive extraction of lignin from wood using supercritical ammonia-water mixtures. *J. Supercrit. Fluids*, 6 (4): 249–254.
56. Shen, J.W.; Domański, K.B.; Kitao, O.; Nakanishi, K. (1995) Computer simulation on supercritical carbon dioxide fluid a potential model for the benzene-carbon dioxide system from ab initio calculations. *Fluid Phase Equilibr.*, 104 (1): 375–390.
57. Olsen, M.H.N.; Salomão, G.C.; Drago, V.; Fernandes, C.; Horn Jr., A.; Cardozo, F.L.; Antunes, O.A.C. (2005) Oxidation of cyclohexane in supercritical carbon dioxide catalyzed by iron tetraphenylporphyrin. *J. Supercrit. Fluids*, 34 (2): 119–124.
58. Glisic, S.B.; Misicb, D.R.; Stamenica, M.D.; Zizovica, I.T.; Asaninb, R.M.; Skala, D.U. (2007) Supercritical carbon dioxide extraction of carrot fruit essential oil: Chemical composition and antimicrobial activity. *Food Chem.*, 105 (1): 346–352.
59. Brunner, G.; Peter, S. (1982) On the solubility of glycerides and fatty acids incompressed gases in the presence of an entrainer. *Sep. Sci. Technol.*, 17 (1): 199–214.
60. Shende, R.V.; Lombardo, S.J. (2002) Supercritical extraction with carbon dioxide and ethylene of poly(vinyl butyral) and dioctyl phthalate from multilayer ceramic capacitors. *J. Supercrit. Fluids*, 23 (2): 153–162.
61. Hamdan, S.; Daood, H.G.; Toth-Markus, M.; Illés, V. (2008) Extraction of cardamom oil by supercritical carbon dioxide and sub-critical propane. *J. Supercrit. Fluids*, 44 (1): 25–30.
62. Banaei, M.; Florusse, L.J.; Raeissi, S.; Peters, C.J. (2012) High-pressure vapor–liquid equilibria of methanol + propylene: Experimental and modeling with SAFT. *J. Supercrit. Fluids*, 63: 25–30.
63. Kershaw, J.R.; Smart, P.J. (1993) Extraction of coal-tar pitch with supercritical toluene. *J. Supercrit. Fluids*, 6 (3): 155–163.
64. Ho, C.H.L.; Cacece, J.E.; Mazza, G. (2007) Extraction of lignans, proteins and carbohydrates from flaxseed meal with pressurized low polarity water. *Food Sci. Technol.*, 40 (9): 1637–1647.
65. Straver, E.J.M.; Roo, J.L.; Peters, C.J. Swaan-Arons, J. (1998) Phase behavior of the binary system propane and tristearin. *J. Supercrit. Fluids*, 11 (3): 139–150.
66. Illés, V.; Daood, H.G.; Perneczki, S.; Szokonya, L.; Then, M. (2000) Extraction of coriander seed oil by CO_2 and propane at superand subcritical conditions. *J. Supercrit. Fluids*, 17 (2): 177–186.
67. Freitas, L.S.; Oliveira, J.V.; Dariva, C.; Jacques, R.A.; Caramão, E.B. (2008) Extraction of grape seed oil using compressed carbon dioxide and propane: extraction yields and characterization of free glycerol compounds. *J. Agr. Food Chem.*, 56 (8): 2558–2564.
68. Zancan, K.C.; Marques, M.O.M.; Petenate, A.J.; Meireles, M.A.A. (2002) Extraction of ginger (*Zingiber officinale roscoe*) oleoresin with CO_2 and co-solvents: A study of the antioxidant activity of the extracts. *J. Supercrit. Fluids*, 24 (1): 57–76.
69. Yoda, S.K.; Marques, M.O.; Petenate, A.J.; Meireles, M.A.A. (2003) Supercritical fluid extraction from *Stevia rebaudiana Bertoni* using CO_2 and CO_2 plus water: extraction kinetics and identification of extracted components. *J. Food Eng.*, 57 (2): 125–134.
70. Sun, Y.; Liu, Z.; Wang, J.; Tian, W.; Zhou, H.; Zhu, L.; Zahng, C. (2008) Supercritical fluid extraction of paeonol from *Cynanchum paniculatum* (Bge.) Kitag. and subsequent isolation by high-speed counter-current chromatography coupled with high-performance liquid chromatography-photodiode array detector. *Sep. Purif. Technol.*, 64 (2): 221–226.
71. You, G.S.; Lin, S.C.; Chen, C.R.; Tsai, W.C.; Chang, C.J.; Huang, W.W. (2002) Supercritical carbon dioxide extraction enhances flavonoids in water-soluble propolis. *J. Chin. Inst. Chem. Eng.*, 33 (3): 233–241.
72. Saldaña, M.D.A.; Zetzl, C.; Mohamed, R.S.; Brunner, G. (2002) Extraction of methylxanthines from guarana seeds, mate leaves, and cocoa beans using supercritical carbon dioxide and ethanol. *J. Agr. Food Chem.*, 50 (17): 4820–4826.
73. Catchpole, O.J.; Grey, J.B.; Mitchel, K.A.; Lan, J.S. (2004) Supercritical antisolvent fractionation of propolis tincture. *J. Supercrit. Fluids*, 29 (1–2): 97–106.
74. Lee, Y.N.; Chen, C.R.; Yang, H.L.; Lin, C.C.; Chang, C.M.J. (2007) Isolation and purification of 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) in Brazilian propolis by supercritical fluid extractions. *Sep. Purif. Technol.*, 54 (1): 130–138.
75. Kitzberger, C.S.G.; Smânia, A.J.; Pedrosa, R.C.; Ferreira, S.R.S. (2007) Antioxidant and antimicrobial activities of shiitake (*Lentinula edodes*) extracts obtained by organic solvents and supercritical fluids. *J. Food Eng.*, 80 (2): 631–638.
76. Chen, C.R.; Lee, Y.N.; Lee, M.R., Chang, C.M.J. (2009) Supercritical fluids extraction of cinnamic acid derivatives from Brazilian propolis and the effect on growth inhibition of colon cancer cells. *J. Taiwan Inst. Chem. Eng.*, 40 (2): 130–135.
77. Chen, C.R.; Shen, C.T.; Wu, J.J.; Yang, H.L., Hsu, S.L.; Chang, C.M.J. (2009) Precipitation of sub-micron particles of 3,5-diprenyl-4-hydroxycinnamic acid in Brazilian propolis from supercritical carbon dioxide anti-solvent solutions. *J. Supercrit. Fluids*, 50 (2): 176–182.
78. Azevedo, A.B.A.; Kopcak, U.; Mohamed, R.S. (2003) Extraction of fat from fermented *Cupuaçu* seeds with supercritical solvents. *J. Supercrit. Fluids*, 27 (2): 223–237.
79. Yépez, B.; Espinosa, M.; López, S.; Bolaños, G. (2002) Producing antioxidant fractions from herbaceaus matrices by supercritical fluid extraction. *Fluid Phase Equilibr.*, 194–197: 879–884.
80. Mohamed, R.S. (1997) Extraction and fractionation of naturally occurring products with supercritical fluids. *Sci. Food Technol.*, 17 (4): 344–353.
81. Dohrn, R.; Brunner, G. (1995) High-pressure fluid-phase equilibria: Experimental methods and systems investigated (1988–1993). *Fluid Phase Equilibr.*, 106 (1–2): 213–282.
82. Moura, L.S.; Carvalho, R.N.; Stefanini, M.B.; Ming, L.C.; Meireles, M.A. (2005) Supercritical fluid extraction from fennel (*Foeniculum vulgare*): global yield, composition and kinetic data. *J. Supercrit. Fluids*, 35 (3): 212–219.
83. Pereira, C.G.; Gualtieri, I.P.; Meireles, M.A.A. (2008) Effect of different extraction processes on the recovery of extracts from *Achyrocline satureioides* D.C.: An evaluation of antioxidant activity. *Sep. Sci. Technol.*, 43 (6): 1549–1563.
84. Braga, M.E.M. (2005) Obtaining bioactive compounds from *Curcuma longa* L. and *Lippia alba* M. by supercritical technology: global yields, extraction kinetics, chemical composition, and usage of the starchy residue. PhD Thesis, School of Food Engineering, University of Campinas, SP, Brazil.
85. Seabra, I.J.; Braga, M.E.M.; Batista, M.T.P.; Sousa, H.C. (2010) Fractioned high pressure extraction of anthocyanins from elderberry (*Sambucus nigra* L.) pomace. *Food Bioprocess Technol.*, 3: 674–683.
86. Yonei, Y.; Ohinata, H.; Yoshida, R.; Shimizu, Y.; Yokoyama, C. (1995) Extraction of ginger flavor with liquid or supercritical carbon dioxide. *J. Supercrit. Fluids*, 8 (2): 156–161.

87. Pereira, C.G.; Marques, M.O.M.; Barreto, A.S.; Siani, A.C.; Fernandes, E.C.; Meireles, M.A.A. (2004) Extraction of indole alkaloids from *Tabernaemontana catharinensis* using supercritical CO_2 + ethanol: An evaluation of the process variables and the raw material origin. *J. Supercrit. Fluids*, 30 (1): 51–61.
88. Setianto, W.B.; Yoshikawa, S.; Smith Jr., R.L.; Inomata, H.; Florusse, L.J.; Peters, C.J. (2009) Pressure profile separation of phenolic liquid compounds from cashew (*Anacardium occidentale*) shell with supercritical carbon dioxide and aspects of its phase equilibria. *J. Supercrit. Fluids*, 48 (3): 203–210.
89. King, J.W. (2000) Advances in critical fluid technology for food processing. *Food Sci. Technol.*, 14 (4): 186–191.
90. Perrut, M.; Clavier, J.Y. (2003) Supercritical fluid formulation: Process cand scale-up. *Ind. Eng. Chem. Res.*, 42 (25): 6375–6383.
91. Meireles, M.A.A. (2003) Supercritical extraction from solid process design data (2001–2003). *Cur. Opin. Solid St. M. Sci.*, 7 (4–5): 321–330.
92. Bhat, V.B.; Madyastha, K.M. (2000) C-Phycocyanin: A potent peroxyl radical scavenger in vivo and in vitro. *Biochem. Bioph. Res. Co.*, 275 (1): 20–25.
93. Piñero-Estrada, J.E.; Bermejo-Bascós, P.; Villar, F.A.M. (2001) Antioxidant activity of different fractions of *Spirulina platensis* protean extract. *Farmaco*, 56 (5–7): 497–500.
94. Magnan, C.; Badens, E.; Commenges, N.; Charbit, G. (2000) Soy lecithin micronization by precipitation with a compressed fluid antisolvent: Influence of process parameters. *J. Supercrit. Fluids*, 19 (1): 69–77.
95. Sun, M.; Temelli, F. (2006) Supercritical carbon dioxide extraction of carotenoids from carrot using canola oil as a continuous co-solvent. *J. Supercrit. Fluids*, 37 (3): 397–408.
96. Bravi, M.; Spinoglio, F.; Verdone, N.; Adami, N.; Aliboni, A.; D'andréa, A.; Santis, D.; Ferri, D. (2007) Improving the extraction of α-tocopherol-enriched oil from grape seeds by supercritical CO_2: Optimisation of the extraction conditions. *J. Food Eng.*, 78 (2): 488–493.
97. Ruivo, R.; Couto, R.; Simoes, P.C. (2008) Supercritical carbon dioxide fractionation of the model mixture squalene/oleic acid in a membrane contactor. *Sep. Purif. Technol.*, 59 (3): 231–237.
98. Stashenko, E.E.; Jaramillo, B.E.; Martínez, J.R. (2004) Comparison of different extraction methods for the analysis of volatile secondary metabolites of *Lippia Alba* (Mill.) N. E. Brown, grown in Colombia, and evaluation of its in vitro antioxidant activity. *J. Chromatogr.*, 1025 (1): 93–103.
99. Li, J.; Zhang, M.; Zheng, T. (2009) The in vitro antioxidant activity of lotus germ oil from supercritical fluid carbon dioxide extraction. *Food Chem.*, 115 (3): 939–944.
100. Ixtaina, V.Y.; Vega, A.; Nolasco, S.M.; Tomás, M.C.; Gimeno, M.; Bárzana, E.; Tecante, A. (2010) Supercritical carbon dioxide extraction of oil from Mexican chia seed (*Salvia hispanica* L.): Characterization and process optimization. *J. Supercrit. Fluids*, 55 (1): 192–199.
101. Ribeiro, M.A.; Bernardo-Gil, M.G.; Esquível, M.M. (2001) *Melissa officinalis L.*: study of antioxidant activity in supercritical residues. *J. Supercrit. Fluids*, 21 (1): 51–60.
102. Yamini, Y.; Khajeh, M.; Ghasemi, E.; Mirza, M.; Javidnia, K. (2008) Comparison of essential oil compositions of *Salvia mirzayanii* obtained by supercritical carbon dioxide extraction and hydrodistillation methods. *Food Chem.*, 108 (1): 341–346.
103. Povh, N.P.; Marques, M.O.M.; Meireles, M.A.A. (2001) Supercritical CO_2 extraction of essential oil and oleoresin from chamomile (*Chamomilla recutita* [L.] *Rauschert*). *J. Supercrit. Fluids*, 21 (3): 245–256.
104. Uribe, J.A.R.; Perez, J.I.N.; Kauil, H.C.; Rubio, G.R.; Alcocer, C.G. (2011) Extraction of oil from chia seeds with supercritical CO_2. *J. Supercrit. Fluids*, 56 (2): 174–178.
105. Kotnik, P.; Skerget, M.; Knez, Z. (2007) Supercritical fluid extraction of chamomile flower heads: Comparison with conventional extraction, kinetics and scale-up. *J. Supercrit. Fluids*, 43 (2): 192–198.
106. Aleksovski, S.A.; Sovova, H. (2007) Supercritical CO_2 extraction of *Salvia officinalis L. J. Supercrit. Fluids*, 40 (2): 139–145.
107. Alhaj, N.A.; Shamsudin, M.N.; Zamri, H.F.; Abdullah, R. (2008) Extraction of essential oil from *Nigella sativa* using supercritical carbon dioxide: Study of antibacterial activity. *Am. J. Pharmacol.*, 3 (4): 225–228.
108. Grigonis, D.; Venskutonis, P.R.; Sivik, B.; Sandahl, M.; Eskilsson, C. (2005) Comparison of different extraction techniques for isolation of antioxidants from sweet grass (*Hierochloe odorata*). *J. Supercrit. Fluids*, 33 (3): 223–233.
109. Ruetsch, L.; Daghero, J.; Mattea, M. (2003) Supercritical extraction of solid matrices. Model formulation and experiments. *Latin Am. Appl. Res.*, 33 (2): 103–107.
110. Ramírez, P.; Senoráns, F.J.; Ibañes, E.; Reglero, G. (2004) Separation of rosemary antioxidant compounds by supercritical fluid chromatography on coated packed capillary columns. *J. Chromatogr.*, 1057 (1–2): 241–245.
111. Goli, A.H.; Barzegar, M.; Sahara, M.A. (2005) Antioxidant activity and total phenolic compounds of pistachio (*Pistachia vera*) hull extracts. *Food Chem.*, 92 (3): 521–525.
112. Quispe-Condori, S.; Foglio, M.A.; Rosa, P.T.V.; Meireles, M.A.A. (2008) Obtaining β-caryophyllene from *Cordia verbenacea* de Candolle by supercritical fluid extraction. *J. Supercrit. Fluids*, 46 (1): 27–32.
113. Donelian, A.; Carlson, L.H.C.; Lopes, T.J.; Machado, R.A.F. (2009) Comparison of extraction of patchouli (*Pogostemon cablin*) essential oil with supercritical CO_2 and by steam distillation. *J. Supercrit. Fluids*, 48 (1): 15–20.
114. Guan, W.; Li, S.; Yan, R.; Tang, S.; Quan, C. (2007) Comparison of essential oils of clove buds extracted with supercritical carbon dioxide and other three traditional extraction methods. *Food Chem.*, 101 (4): 1558–1564.
115. Cao, H.; Xiao, J.B.; Xu, M. (2007) Comparison of volatile components of *Marchantia convoluta* obtained by supercritical carbon dioxide extraction and petrol ether extraction. *J. Food Compos. Anal.*, 20 (1): 45–51.
116. Vargas, C.E.; Mendes, M.F.; Azevedo, D.A.; Pessoa, F.L.P.; Uller, A.C. (2010) Extraction of the essential oil of abajeru (*Chrysobalanus icaco*) using supercritical CO_2. *J. Supercrit. Fluids*, 54 (2): 171–177.
117. Özcan, A.; Özcan, A.S. (2004) Comparison of supercritical fluid and Soxhlet extractions for the quantification of hydrocarbons from Euphorbia macroclada. *Talanta*, 64 (2): 491–4958.
118. Felfoldi-Gáva, A.; Szarka, S.; Simándi, B.; Blazics, B.; Simon, B.; Kéry, A. (2012) Supercritical fluid extraction of *Alnus glutinosa* (L.) Gaertn. *J. Supercrit. Fluids*, 61: 55–61.
119. Glisic, S.; Ivanovic, J.; Ristic, M.; Skala, D.U. (2010) Extraction of sage (*Salvia officinalis* L.) by supercritical CO_2: Kinetic data, chemical composition and selectivity of diterpenes. *J. Supercrit. Fluids*, 52 (1): 62–70.
120. Marcucci, M.C.; Ferreres, F.; Guarcía-Viguera, C.; Bankova, V.S.; De Castro, S.L.; Dantas, A.P.; Valente, P.H.M.; Paulino, N. (2001) Phenolic compounds from Brazilian própolis with pharmacollogical activities. *J. Ethnopharmacol.*, 74 (2): 105–112.
121. Park, Y.K.; Alencar, S.M.; Scamparini, A.R.P.; Aguiar, C.L. (2002) Propolis produced in South Brazil, Argentine and Uruguay: Phytochemical evidence for the plant origin. *Rural Sci.*, 32 (6): 997–1003.
122. Cunha, I.B.S.; Sawaya, A.C.H.F.; Caetano, F.M.; Shimizu, M.T.; Marcucci, M.C.; Drezza, F.T.; Povia, G.S.; Carvalho, P.O. (2004) Factors that Influence the yield and composition of Brazilian propolis extracts. *J. Braz. Chem. Soc.*, 15 (6): 964–970.

123. Nagai, T.; Inoue, R.; Inoue, H.; Suzuki, N. (2003) Preparation and antioxidant properties of water extract of propolis. *Food Chem.*, 80 (1): 29–33.
124. Kumazawa, S.; Hamasaka, T.; Nakayama, T. (2004) Antioxidant activity of propolis of various geographic origin. *Food Chem.*, 84 (3): 329–339.
125. Wang, B.J.; Lien, Y.H.; Yu, Z.R. (2004) Supercritical fluid extractive fractionation study of the antioxidant activities of propolis. *Food Chem.*, 86 (2): 237–243.
126. Lu, L.C.; Chen, Y.W.; Chou, C.C. (2005) Antibacterial activity of propolis against *Staphylococcus aureus*. Inter. *J. Food Microbiol.*, 102 (2): 213–220.
127. Antungulu, G.G.; Toshitaka, U.; Fumihiko, T.; Daisuke, H. (2008) Effect of vapors from fractionated samples of propolis on microbial and oxidation damage of rice during storage. *J. Food Eng.*, 88: 341–352.
128. Paulino, N.; Abreu, S.R.L.; Uto, Y.; Koyama, D.; Nagasawa, H.; Hori, H.; Dirsch, V.M.; Vollmar, A.M.; Scremin, A.; Bretz, W.A. (2008) Anti-inflamatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. *Eur. J. Pharmacol.*, 587 (1–3): 296–301.
129. Ito, J.; Chang, F.R.; Wang, H.K.; Park, Y.K.; Ikegaki, M.; Kilgore, N.; Lee, K.H. (2001) Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian própolis. *J. Nat. Prod.*, 64 (10): 1278–1281.
130. Wu, J.; Luyu, Q. Supercritical multiple extraction of bee glue. Chinese Patent, 1258511, 2000 July 05.
131. Paviani, C.L.; Dariva, C.; Marcucci, M.C.; Cabral, F.A. (2010) Surpercritical cabon dioxide selectivity to fracionate phenolic compounds from the dry ethanolic extract of propolis. *J. Food Proc. Eng.*, 33 (1): 15–27.
132. Abbasi, H.; Rezaei, K.; Radishi, L. (2008) Extraction of essential oils from the seeds of pomegranate using organic solvents and supercritical CO_2. *J. Am. Chem. Soc.*, 85 (1): 83–89.
133. Martínez, M.L.; Mattea, M.A.; Maestri, D.M. (2008) Pressing and supercritical carbon dioxide extraction of walnut oil. *J. Food Eng.*, 88 (3): 399–404.
134. Sánchez-Vicente, Y.; Cabañas, A.; Renuncio, J.A.R.; Pando, C. (2009) Supercritical fluid extraction of peach (*Prunus persica*) seed oil using carbon dioxide and ethanol. *J. Supercrit. Fluids*, 49 (2): 167–173.
135. Bhattacharjee, P.; Singhal, R.S.; Tiwari, S.R. (2007) Supercritical carbon dioxide extraction of cottonseed oil. *J. Food Eng.*, 79 (3): 892–898.
136. Gomez-Pietro, M.S.; Caja, M.M.; Herraiz, M.; Santa-Maria, G. (2003) Supercritical fluid extraction of all-trans-Lycopene from Tomato. *J. Agric. Food Chem.*, 51 (1): 3–7.
137. Vági, E.; Simándi, B.; Vásárhelyine, K.P.; Daood, H.; Kéry, A.; Doleschall, F.; Nagy, B. (2007) Supercritical carbon dioxide extraction of carotenoids, tocopherols and sitosterols from industrial tomato by-products. *J. Supercrit. Fluids*, 40 (2): 218–226.
138. Montañés, F.; Fornari, T.; Martín-Álvarez, P.J.; Montilla, A.; Corzo, N.; Olano, A.; Ibáñes, J. (2007) Selective fractionation of disaccharide mixtures by supercritical CO_2 with ethanol as co-solvent. *J. Supercrit. Fluids*, 41 (1): 61–67.
139. Montañés, F.; Olano, A.; Reglero, G.; Ibáñez, E.; Fornari, T. (2009) Supercritical technology as an alternative to fractionate prebiotic galactooligosaccharides. *Sep. Purif. Technol.*, 66 (2): 383–389.
140. Perretti, G.; Motori, A.; Bravi, E.; Favati, F.; Montanari, L.; Fantozzi, P. (2007) Supercritical carbon dioxide fractionation of fish oil fatty acid ethyl esters. *J. Supercrit. Fluids*, 40 (3): 349–353.
141. Chang, L.H.; Shen, C.T.; Hsieh, S.J.; Hsu, S.L.; Chang, H.C.; Chang, C.M.J. (2008) Recovery and enhancement of unsaturated fatty acids in soft-shelled turtle fish oil using supercritical carbon dioxide and associated catalase release activity. *Separation and Purification Technol.*, 64 (2): 213–220.
142. Davanejad, K.M.; Kassim, A.; Zainal, A.; Sata, S.A. (2008) Supercritical fluid extraction of β-carotene from crude palm oil using CO_2. *J. Food Eng.*, 89 (4): 472–478.
143. Espinosa, S.; Díaz, M.S.; Brignole, E.A. (2008) Food additives obtained by supercritical extraction from natural sources. *J. Supercrit. Fluids*, 45 (2): 213–219.
144. Rubio-Rodríguez, N.; Diego, S.M.; Beltrán, S.; Jaime, I.; Sanz, M.T.; Rovira, J. (2012) Supercritical fluid extraction of fish oil from fish by-products: A comparison with other extraction methods. *J. Food Eng.*, 109 (2): 238–248.
145. Bernal, J.L.; Nozal, M.J.; Toribio, L.; Diego, C.; Mayo, R.; Maestre, R. (2008) Use of supercritical fluid extraction and gas chromatography-mass spectrometry to obtain amino acid profiles from several genetically modified varieties of maize and soybean. *J. Chromatogr. A*, 1192 (2): 266–272.
146. Toribio, L.; Berbal, J.L.; Nozal, M.J.; Arnaiz, E.; Bernal, J. (2011) Sequential supercritical fluid extraction of lipids application to the obtention of the fatty acid profile of some genetically modified varieties of corn. *Food Anal. Method.*, 4 (2): 196–202.
147. Ozkal, S.G.; Salgin, U.; Yener, M.E. (2005) Supercritical carbon dioxide extraction of hazelnut oil. *J. Food Eng.*, 69 (2): 217–223.
148. Danielski, L.; Zetzl, C.; Hense, H.; Brunner, G. (2005) A process line for the production of raffinated rice oil from rice bran. *J. Supercrit. Fluids*, 34 (2): 133–141.
149. Saldaña, M.D.A.; Mohamed, R.S.; Mazzafera, P. (2002) Extraction of cocoa butter from Brazilian cocoa beans using supercritical CO_2 and ethane. *Fluid Phase Equilibr.*, (194–197): 885–894.
150. Saengcharoenrat, C.; Guyer, D.E. (2004) Effects of supercritical carbon dioxide conditions on onion oil desorption. *J. Food Eng.*, 63 (1): 33–37.
151. Doker, O.; Salgin, U.; Sanal, I.; Mehmetoglu, Ü.; Çalimli, A. (2004) Modeling of extraction of β-carotene from apricot bagasse using supercritical CO_2 in packed bed extractor. *J. Supercrit. Fluids*, 28 (1): 11–19.
152. Salgin, U.; Doker, O.; Çalimli, A. (2006) Extraction of sunflower oil with supercritical CO_2: Experiments and modeling. *J. Supercrit. Fluids*, 38 (3): 326–331.
153. Berna, A.; Tárregab, A.; Blaco, M.; Subirats, S. (2000) Supercritical CO_2 extraction of essential oil from orange peel; effect of the height of the bed. *J. Supercrit. Fluids*, 18 (3): 227–237.
154. França, L.F.; Meireles, M.A. (2000) Modeling the extraction of carotene and lipids from pressed palm oil (*Elaes guineensis*) fibers using supercritical CO_2. *J. Supercrit. Fluids*, 18 (1): 35–47.
155. Del-Valle, J.M.; Jimenez, M.; La-Fuente, J.C. (2003) Extraction kinetics of pre-pelletized Jalapeño peppers with supercritical CO_2. *J. Supercrit. Fluids*, 25 (1): 33–44.
156. Louli, V.; Folas, G.; Voutsas, E.; Magoulas, K. (2004) Extraction of parsley seed oil by supercritical CO_2. *J. Supercrit. Fluids*, 30 (2): 163–174.
157. Campos, L.M.A.S.; Leimann, F.V.; Pedrosa, R.C.; Ferreira, S.R.S. (2008) Free radical scavenging of grape pomace extracts from cabernet sauvingnon (*Vitis vinifera*). *Bioresour. Technol.*, 99 (17): 8413–8420.
158. Passos, C.P.; Silva, R.M.; Silva, F.A.; Coimbra, M.A.; Silva, C.M. (2009) Enhancement of the supercritical fluid extraction of grape seed oil by using enzymatically pre-treated seed. *J. Supercrit. Fluids*, 48 (3): 225–229.
159. Vasapollo, G.; Longo, L.; Restio, L.; Ciurlia, L. (2004) Innovative supercritical CO_2 extraction of Lycopene from tomato in the presence of vegetable oil as co-solvent. *J. Supercrit. Fluids*, 29 (1–2): 87–96.
160. Sanal, I.S.; Bayraktar, E.; Mehmetoglu, U.; Calimli, A. (2005) Determination of optimum conditions for SC-(CO_2 + ethanol) extraction of β-carotene from apricot pomace using response surface methodology. *J. Supercrit. Fluids*, 34 (3): 331–338.

161. Moore, S.; Samdani, S.; Ondrey, G.; Parkinson, G. (1994) New roles for supercritical. *Chem. Eng.*, 32–35.
162. Kim, W.J.; Kim, J.D.; Kim, J.; Oh, S.G.; Lee, Y.W. (2008) Selective caffeine removal from green tea using supercritical carbon dioxide extraction. *J. Food Eng.*, 89 (3): 303–309.
163. Sun, Q.L.; Shu, H.; Ye, J.H.; Lu, J.L.; Zheng, X.Q.; Liang, Y.R. (2010) Decaffeination of green tea by supercritical carbon dioxide. *J. Med. Plant. Res.*, 4 (12): 1161–1168.
164. Perretti, G. (2006) Supercritical Carbon Dioxide Extraction of Agricultural and Food Processing Wastes and Byproducts. *Mod. Ext. Tech. Am. Chem. Soc.*, 926 (2): 23–35.
165. De-Lucas, E.; Martínez, O.; Rincon, J.; Blanco, M.A.; Gracia, I. (2002) Supercritical fluid extraction of tocopherol concentrates from olive tree leaves. *J. Supercrit. Fluids*, 22 (3): 221–228.
166. Machmudah, S.; Winardi, S.; Sasaki, M.; Goto, M.; Kusumoto, N.; Hayakawa, K. (2012) Lycopene extraction from tomato peel by-product containing tomato seed using supercritical carbon dioxide. *J. Food Eng.*, 108 (2): 290–296.
167. Klejdus, B.; Lojková, L.; Plaza, M.; Šnóblová, M.; Šterbová, D. (2010) Hyphenated technique for the extraction and determination of isoflavones in algae: Ultrasound-assisted supercritical fluid extraction followed by fast chromatography with tandem mass spectrometry. *J. Chromatogr A*, 1217 (51): 7956–7965.
168. Patil, P.D.; Gude, V.G.; Mannarswamy, A.; Deng, S.; Cooke, P.; Munson-Mcgee, S.; Rhodes, I.; Lammers, P.; Nirmalakhandan, N. (2011) Optimization of direct conversion of wet algae to biodiesel under supercritical methanol conditions. *Bioresour. Technol.*, 102 (1): 118–122.
169. Pasquali, I. Bettini, R. (2008) Are pharmaceutics really going supercritical? *International J. Pharmaceut.*, 364 (2): 176–187.
170. Lesoin, L.; Crampon, C. Boutin, O. Badens, E. (2011) Preparation of liposomes using the supercritical anti-solvent (SAS) process and comparison with a conventional method. *J. Supercrit. Fluids*, 57 (2): 162–174.
171. Sunarso, J.; Ismadji, S. (2009) Decontamination of hazardous substances from solid matrices and liquids using supercritical fluid extraction: A review. *J. Hazard. Mater.*, 161 (1): 1–20.
172. Chen, C.H.; Chen, W.H.; Chang, C.M.J.; Lai, S.M.; Tu, C.H. (2010) Biodiesel production from supercritical carbon dioxide extracted *Jatropha* oil using subcritical hydrolysis and supercritical methylation. *J. Supercrit. Fluids*, 52 (2): 228–234.
173. Lim, S. Hoong, S.S.; Teong, L.K.; Bhatia, S. (2010) Supercritical fluid reactive extraction of *Jatropha curcas L.* seeds with methanol: A novel biodiesel production method. *Bioresour. Technol.*, 101 (18): 7169–7172.
174. Breet, E.; Nortjé, Y.; Greuning, C. (2011) Supercritical carbon dioxide extracted oil from *Jatropha curcas*: Directive for the biodiesel industry? *J. Supercrit. Fluids*, 60: 38–44.
175. Lim, S.; Lee, K.T. (2011) Effects of solid pre-treatment towards optimizing supercritical methanol extraction and transesterification of *Jatropha curcas L.* seeds for the production of biodiesel. *Sep. Purif. Technol.*, 81 (3): 363–370.
176. Taher, H.; Al-Zuhair, S. Al-Marzouqui, A.; Hashim, I. (2011) Extracted fat from lamb meat by supercritical CO_2 as feedstock for biodiesel production. *Biochem. Engn. J.*, 55 (1): 23–31.
177. Al-Zuhair, S.; Hussein, A.; Al-Marzouqi, A.H.; Hashim, I. (2012) Continuous production of biodiesel from fat extracted from lamb meat in supercritical CO_2 media. *Biochem. Engn. J.*, 60 (15): 106–110.
178. Gui, M.M.; Lee, K.T.; Bhatia, S. (2009) Supercritical ethanol technology for the production of biodiesel: Process optimization studies. *J. Supercrit. Fluids*, 49 (2): 286–292.
179. Vezzú, K.; Sforza, E.; Sudiro, M.; Beneditti, P.; Losasso, C.; Bertucco, A. (2009) Production of bioethanol under high pressure of CO_2: The effect of process conditions. *J. Supercrit. Fluids*, 51 (1): 67–73.
180. Arias, M.; Penichet, I.; Ysambertt, F.; Bauza, R.; Zougagh, M. Ríos, A. (2009) Fast supercritical fluid extraction of low- and high-density polyethylene additives: Comparison with conventional reflux and automatic Soxhlet extraction. *J. Supercrit. Fluids*, 50 (1): 22–28.
181. Areerat, S.; Hayata, Y.; Katsumoto, R.; Kegasawa, T.; Egami, H.; Ohshima, M. (2004) Measurement and prediction of diffusion coefficient of supercritical CO_2 in molten polymer. *Pol. Eng. Sci.*, 44 (10): 1915–24.
182. Fujiwara, T.; Yamaoka, K.Y.; Wynne, K.J. (2005) Poly (lactide) swelling and melting behavior in supercritical carbon dioxide and post-venting porous material. *Biomacromol.*, 6: 2370–3.
183. Furlan, M.; Kluge, J.; Mazzotti, M.; Lattuada, M. (2010) Preparation of biocompatible magnetite–PLGA composite nanoparticles using supercritical fluid extraction of emulsions. *J. Supercrit. Fluids*, 54 (3): 348–356.
184. Owen, R.; Lewis, A.L.; Whitaker, M.J.; Tai, H.; Shakesheff, K.M.; Howdle, S.M. (2008) Applications of supercritical CO_2 in the fabrication of polymer systems for drug delivery and tissue engineering. *Adv. Drug Deliver. Rev.*, 60 (3): 373–387.
185. Rezaee, M.; Yamini, Y.; Moradi, M.; Saleh, A.; Faraji, M.; Naeeni, M.H. (2010) Supercritical fluid extraction combined with dispersive liquid–liquid microextraction as a sensitive and efficient sample preparation method for determination of organic compounds in solid samples. *J. Supercrit. Fluids*, 55: 161–168.
186. Naeeni, M.H.; Yamini, Y.; Rezaee, M. (2011) Combination of supercritical fluid extraction with dispersive liquid–liquid microextraction for extraction of organophosphorus pesticides from soil and marine sediment samples. *J. Supercrit. Fluids*, 57: 219–226.
187. Yang, T.J.; Tsai, F.J.; Chen, C.Y.; Yang, T.C.C.; Lee, M.R. (2010) Determination of additives in cosmetics by supercritical fluid extraction on-line headspace solid-phase microextraction combined with gas chromatography–mass spectrometry. *Anal. Chim. Acta.*, 668: 188–194.
188. Klejdus, B.; Kopecky, J.; Benesová, L.; Vacek, J. (2009) Solid-phase/supercritical-fluid extraction for liquid chromatography of phenolic compounds in freshwater microalgae and selected cyanobacterial species. *J. Chromatogr A*, 1216: 763–771.
189. Melo, C.I.; Bogel-Łukasika, R.; Bogel-Łukasik, E. (2012) Combination of supercritical carbon dioxide and ionic liquid in a novel assembly of carvacrol. *J. Supercrit. Fluids*, 61: 191–198.
190. Glisic, S.B.; Ristic, M.; Skala, D.U. (2011) The combined extraction of sage (*Salvia officinalis* L.): Ultrasound followed by supercritical CO_2 extraction. *Ultrason. Sonochem.*, 18: 318–326.
191. Danielski, L.; Brunner, G.; Schwanke, C.; Zetzl, C.; Hense, H.; Donoso, J.P.M. (2008) Deterpenation of mandarin (*Citrus reticulata*) peel oils by means of countercurrent multistage extraction and adsorption/desorption with supercritical CO_2. *J. Supercrit. Fluids*, 44: 315–324.
192. Ortuño, C.; Martínez-Pastor, M.T.; Mulet, A.; Benedito, J. (2013) Application of high power ultrasound in the supercritical carbon dioxide inactivation of *Saccharomyces cerevisiae*. *Food Res. Int.*, 51 (2): 474–481.
193. Ortuño, C.; Martínez-Pastor, M.T.; Mulet, A.; Benedito, J. (2012) An ultrasound-enhanced system for microbial inactivation using supercritical carbon dioxide. *Innov. Food Sci. Emerg.*, 15: 31–37.
194. Adams, M.K. (1991) Analysis of coffee: production and trade. *Allentown: Liquid Carbonic Industries Corporation*, pp. 1–30, 1991.
195. Anklam, E.; Müller, A. (1995) Extraction of caffeine and vanillin from drugs by supercritical carbon dioxide. *Pharmazie*, 50 (1): 364–365.
196. Mcnally, M.E.P. (1996) Method development in supercritical fluid extraction. *J. AOAC Inter.*, 79 (4): 380–387.

Capítulo 4. Artigo

5.0 CAPÍTULO 4: Levantamento dos estudos com a própolis produzida no estado da Bahia

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Levantamento dos estudos com a própolis produzida no estado da Bahia

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Resumo – Própolis é um material de consistência e coloração variada, elaborado por abelhas, a partir de matéria-prima coletada de diversas partes da plantas. Possui uma composição química complexa, a qual depende da flora da região; assim, existem vários tipos de própolis, sendo difícil compará-las. Neste trabalho, é apresentado um levantamento dos estudos realizados com a própolis produzida no estado da Bahia. Foram identificados 17 trabalhos científicos e oito documentos de patentes que tratam da própolis baiana, e elencados três tipos de própolis, dentre as 13 relatadas no Brasil. A maioria dos estudos relatam o potencial da atividade antimicrobiana da própolis do tipo 6, destacando-se seu baixo teor de flavonoides. Verificou-se também o incentivo que está sendo dado à produção da própolis tipo 13 na Bahia, também denominada de própolis vermelha, devido principalmente ao grande valor agregado a este produto.
Palavras-chave adicionais: atividade antimicrobiana, apicultura, documentos de patentes.

Abstract (Survey of studies with propolis produced in the state of Bahia, Brazil) – Propolis is a product of varied consistency and colour, produced by bees from materials collected from various parts of the plants. It has a complex chemical composition, which depends on the regional flora. Therefore, there are various types of propolis, making comparison between them a difficult task. In the present study, a survey of studies with propolis from the state of Bahia, Brazil, was carried out. We identified 17 scientific papers and eight patents dealing with propolis produced in Bahia. In these works three amongst the 13 types of propolis reported in Brazil were listed. Almost all studies report the antimicrobial activity potential of propolis types 6, highlighting its low percentage of flavonoids. It was also noted that the incentive given to the production of propolis type 13 in Bahia, also known as "própolis vermelha", is due to the overall aggregate value of this product.
Additional key words: antimicrobial activity, apiculture, patents documentation.

Própolis é o produto oriundo de substâncias resinosas, gomosas e balsâmicas coletadas por abelhas de diversas espécies, como *Scaptotrigona* aff. *postica* Latreille, 1807 (Souza et al. 2012), *Tetragona clavipes* Fabricius, 1804 e *Melipona mondury* Smith, 1863 (Freitas et al. 2012), *Tetragonisca angustula angustula* Latreille, 1811 e *Trigona recursa* Smith, 1863 (Barth 2006) e *Apis mellifera* Linnaeus, 1758. As fontes de coleta de material são brotos, flores e exudatos de plantas, nos quais são acrescentados secreções salivares, cera e pólen para a elaboração final da própolis (Brasil 2001). As secreções salivares que são acrescentadas aos materiais coletados pelas abelhas contêm a enzima β-glicosidase, acarretando a hidrólise dos flavonoides glicosilados em flavonoides agliconas (Park et al. 1988; Burdock 1998).

O espectro de voo de uma abelha é bastante extenso – o da *Apis mellifera*, por exemplo, abrange um raio de cerca de 4 a 5 km em torno da colmeia – e a composição da própolis é um reflexo direto da flora utilizada pelas abelhas (Adelmann 2005). A cor, sabor, odor, consistência, composição química e atividade biológica da própolis dependem principalmente das espécies vegetais que lhes deram origem e da época do ano em que foram produzidas (Paulino 2004). Dessa maneira, a alteração do pasto apícola e mudanças climáticas podem afetar a composição química da própolis, dificultando sua padronização para comercialização. A variação sazonal, por exemplo, pode implicar a diminuição de alguns componentes biologicamente ativos e o aumento de outros (Nunes et al. 2009).

As abelhas utilizam a própolis para vedar frestas e diminuir o tamanho da entrada da colmeia, reduzindo o ataque de intrusos e protegendo a colmeia e suas crias do frio. Serve ainda como material antisséptico, sendo depositada no interior dos alvéolos onde a abelha rainha realiza a postura dos ovos e também é utilizada para envolver inimigos abatidos no interior da colmeia, evitando que apodreçam e contaminem o ninho (Breyer 1982). Segundo Teixeira et al. (2005), não são conhecidos os fatores que direcionam a preferência das abelhas coletoras de resina por uma determinada fonte botânica, mas sabe-se que elas são seletivas nesta coleta.

Dentre as várias formas de utilização de produtos naturais na medicina, destacam-se as plantas brutas, como ervas, além das tradicionais preparações galênicas, como os extratos (Pereira et al. 2002). A própolis é utilizada como complemento alimentar e medicamento. As propriedades biológicas e terapêuticas da própolis têm sido reconhecidas e comprovadas: atividade antimicrobiana (Bittencourt 2008), anti-inflamatória (Moura et al. 2009), cicatrizante (Souza et al. 2009), antiulcerogênica (Barros et al. 2008), antiparasitária (Pontin et al. 2008) e antioxidante (Oldoni et al. 2011).

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São descritas distintas propriedades biológicas e composições químicas para as amostras de própolis coletadas no Brasil, o que é explicado pela grande biodiversidade brasileira (Pereira et al. 2002). Diante da importância econômica e científica aplicada à própolis nos últimos anos, este trabalho teve como objetivo principal caracterizar a própolis produzida no estado da Bahia sob os aspectos físico-químicos, as atividades biológicas e aplicações, a partir do levantamento de trabalhos publicados em bases de dados de artigos científicos e patentes. Foi realizada uma pesquisa nas bases de dados científicos, como *Science Direct*, *Wiley Online Library*, *SciELO* (*Scientific Electronic Library Online*), e base de dados de documentos de patentes, como a do Instituto Nacional de Propriedade Industrial (INPI), que compila o acervo de todos os documentos de patente de invenção e modelo de utilidade depositados no Brasil. Para o levantamento dos principais trabalhos publicados na área de interesse, utilizou-se uma combinação de palavras-chave, a saber: 'própolis Bahia', 'própolis grupo 6', 'própolis grupo 7' e 'própolis grupo 12', não havendo limite cronológico, sendo selecionados todos os trabalhos identificados que utilizaram própolis oriunda da Bahia.

PRÓPOLIS NO BRASIL

Comercialmente, a própolis tem ocupado lugar de destaque no mercado nacional e internacional de produtos apícolas. Tal inserção deve-se essencialmente à constatação das diferentes atividades biológicas atribuídas aos constituintes químicos desse produto (Teixeira et al. 2003). O Brasil é um dos principais produtores mundiais de própolis, com uma produção estimada em torno de 50 a 150 toneladas por ano, sendo que cerca de 75% desse total é exportado, especialmente para o Japão (97% das exportações) (Lima 2008). No Brasil, já são mais de 10.000 produtores desse produto apícola, sendo mais de 4.000 produtores da própolis verde, exportando anualmente em torno de US$ 30.000.000,00 do produto na forma de extrato alcoólico ou aquoso, encapsulado, associado a outros vegetais também antioxidantes ou simplesmente na forma bruta (Nascimento Junior 2007). Em julho/2010, por exemplo, as exportações de própolis geraram uma receita de US$ 46.417,00 (SEBRAE 2010).

A própolis é utilizada na veterinária para cicatrizar de feridas, em cortes pós-operatórios, controle de hemorragias e tratamento de mastite. Na agricultura, ela é aplicada no tratamento de doenças de algumas espécies vegetais, substituindo produtos químicos, enquanto na indústria de cosméticos, é utilizada como constituinte de cremes de beleza, pasta dental, shampoos e sabonetes. Na indústria de alimentos, é um ingrediente funcional de pastilhas, bombons e chicletes (Costa & Oliveira 2005). No Brasil, uma enorme quantidade de marcas e produtos feitos à base de própolis encontra-se disponível em lojas de produtos naturais, tais como balas, chocolates, doces, chá, protetor solar, gel pós-barba, shampoos, cremes para pele, soluções antissépticas, pastas de dente, sabonetes, batom, cápsulas, extratos, *spray* bucal, pastilhas, xaropes, comprimidos etc. (Park & Ikegaki 1998; Lengler 2012).

A própolis é considerada uma das misturas mais heterogêneas encontradas em fontes naturais; mais de 300 constituintes já foram identificados e/ou caracterizados em diferentes amostras (Lima 2006; Sousa et al. 2007; Lustosa et al. 2008). Dadas às dimensões continentais do Brasil e a grande diversidade da flora e variedade de ecossistemas, sua composição química é extremamente complexa e são vários os tipos de própolis produzidos no país (Lima 2006). Ela pode ser produzida no Brasil todo, porém a qualidade varia de uma região para outra em função de fatores ambientais.

Park et al. (2000) classificaram as amostras de própolis coletadas em todas as Regiões do país (exceto Região Norte) em 12 grupos, de acordo com a aparência e coloração dos extratos (Tabela 1). Posteriormente, foi encontrada uma nova própolis em colmeias localizadas ao longo do litoral e manguezais no nordeste brasileiro, classificada como própolis do grupo 13, denominada própolis vermelha. Observou-se que abelhas coletavam o exsudato vermelho da superfície de *Dalbergia ecastophyllum* (L.) Taub. (Fabaceae), sugerindo que essa seja a origem botânica da própolis vermelha (Daugsch et al. 2006) e análises cromatográficas verificaram que as amostras de própolis e os exudatos resinosos vermelhos apresentam perfis muito semelhantes em relação ao teor de flavonoides (Daugsch et al. 2008).

PRÓPOLIS DA BAHIA

Já está definido na literatura (Park & Ikegaki 1998; Park et al. 2000; Paulino 2004; Adelmann 2005) que a composição química da própolis depende principalmente da flora da região, uma vez que as abelhas coletam o material resinoso das plantas que circundam a colmeia para a produção da própolis. Essa é a principal justificativa para a grande diversidade e quantidade de estudos com diferentes amostras desse material. No presente levantamento bibliográfico foram encontrados 17 trabalhos e oito documentos de patentes que elencam a própolis produzida na Bahia, com a detecção de três tipos de própolis: tipo 6 (marrom-avermelhada), tipo 7 (marrom-esverdeada) e tipo 13 (vermelha).

Segundo os trabalhos de Park et al. (2000) e Alencar (2002), nos quais a própolis brasileira foi classificada em 12 grupos, as amostras da Bahia

Tabela 1. Agrupamento da própolis brasileira quanto à cor. Local de coleta: RS- Rio Grande do Sul; PR- Paraná; BA- Bahia; PE- Pernambuco; CE- Ceará; PI- Piauí; SP- São Paulo). Fonte: Park et al. (2000).

Extrato Etanólico de Própolis			
Grupos*	Cor	Substâncias Solúveis (%)	Origem da própolis
Grupo 1 (RS)	Amarelo	63,0	Região Sul
Grupo 2 (RS)	Castanho Claro	57,0	Região Sul
Grupo 3 (PR)	Castanho Escuro	65,0	Região Sul
Grupo 4 (PR)	Castanho Claro	54,5	Região Sul
Grupo 5 (PR)	Marrom esverdeado	58,7	Região Sul
Grupo 6 (BA)	Marrom avermelhado	45,9	Região Nordeste
Grupo 7 (BA)	Marrom esverdeado	43,8	Região Nordeste
Grupo 8 (PE)	Castanho Escuro	41,3	Região Nordeste
Grupo 9 (PE)	Amarelo	46,7	Região Nordeste
Grupo 10 (CE)	Amarelo Escuro	24,1	Região Nordeste
Grupo 11 (PI)	Amarelo	23,1	Região Nordeste
Grupo 12 (SP)	Verde ou marrom esverdeado	61,0	Região Sudeste

(grupos 6 e 7) foram coletadas nas cidades de Anagé, Barra da Choça, Entre Rios, Jacobina, Juazeiro, Remanso, Salvador, Senhor do Bonfim e Vitória da Conquista, não o grupo de própolis produzido em cada cidade. Nesses estudos, a própolis do tipo 6 se destacou por ter apresentado um perfil fotoquímico não encontrado nas outras própolis estudadas. Além disso, notou-se também um grande incentivo do governo estadual para aumentar a produção da própolis vermelha, principalmente na região Sul da Bahia, onde se concentra uma grande área de manguezais, locais bastante favoráveis para a coleta de resina para a produção da própolis vermelha. A atual produção dessa própolis ainda é considerada pequena, apenas 200 kg por ano sendo esse incentivo justificado pelo grande valor agregado à própolis vermelha, que pode ser vendida por até R$ 450,00 o kg (SECOM 2011).

Korn et al. (2013) avaliaram traços de elementos minerais em 48 amostras de própolis coletadas em diferentes regiões da Bahia (Chapada Diamantina, Litoral Norte, Oeste, Recôncavo, Sudoeste e Sul). Foi verificado que havia diferenças nos teores encontrados para alguns elementos, a saber, Na, Ba, Ca, Cu, Fe, K, Mg, Mn, Ni e Zn. Através da Análise de Componentes Principais (PCA) foi evidenciada a formação de três grupos (A, B e C). O grupo A reuniu amostras relativas à própolis da região da Chapada Diamantina, enquanto os grupos B e C, de localidades próximas (Litoral Norte) e distantes do litoral, respectivamente. Foi observado que os elementos Ni, Zn e K foram mais influentes na separação do grupo A, enquanto Ba foi importante para o grupo B e os elementos Cu, Fe, Mg e Mn para a discriminação do grupo C. Os valores mais sifnigicativos foram o de magnésio e de ferro, superiores nas amostras coletadas do Litoral Norte.

Os diversos estudos feitos com própolis da Bahia concentram-se naquela do tipo 6, com o intuito principal de se verificar a atividade antimicrobiana. Esse tipo de própolis ganhou destaque na área científica depois que diversas pesquisas encontraram um perfil fitoquímico diferenciado, principalmente em relação ao teor de flavonoides (Koo et al. 2000; Moura 2000; Alencar 2002; Duarte 2002). Koo et al. (2000) demonstraram características bastante particulares desta própolis, como alta atividade antimicrobiana e composição química caracterizada por componentes apolares e ausência de flavonoides anglicanas. Os flavonoides são considerados os principais compostos responsáveis pelos efeitos benéficos da própolis. Esses compostos fenólicos provenientes de plantas agem em diferentes processos fisiológicos e exercem função antimicrobiana e antioxidante (Barbosa et al. 2009).

Há grande controvérsia em relação ao verdadeiro teor de flavonoides nas amostras de própolis do Brasil (Pereira et al. 2002). Estudos realizados por Bankova et al. (1995) e Sousa et al. (2007) concluíram que as amostras de própolis brasileira apresentaram baixos teores de flavonoides totais e ésteres de ácidos fenólicos, possuindo altas concentrações de ácido dihidroxicinâmico, acetofenonas preniladas e alguns terpenoides específicos. Como consequência destas diferenças na composição química da própolis, é observada também uma variação nas suas propriedades farmacológicas (Menezes 2005), tendo em vista principalmente o teor dos compostos bioativos presentes nas amostras. Diante de tal diferença, foi criada a Instrução Normativa nº 3, de 19 de janeiro de 2001 para regulamentar o produto apícola que, nos anexos VI e VII de sua resolução, determina os padrões para fixação de identidade e qualidade de própolis e regulamenta a identidade e qualidade de extratos de própolis proveniente da extração dos componentes solúveis em álcool neutro (Brasil 2001). É determinado que estes produtos devam possuir teor mínimo de flavonoides de 0,5% (m/m) e 0,25% (m/m) para própolis e extrato de própolis, respectivamente.

Um estudo bastante amplo em relação aos aspectos físico-químicos e biológicos das própolis produzidas pela abelha *Apis mellifera* na Região Nordeste do Brasil foi realizado por Moura (2000). Das 12 amostras estudadas, nove eram provenientes do estado da Bahia. Mais uma vez, foi observado que uma das amostras da Bahia, mais especificamente da Mata Atlântica e do tipo 6 (coletadas na cidade de Salvador e Entre Rios), apresentava um perfil cromatográfico não encontrado em nenhuma própolis brasileira estudada até então, possuindo compostos de características mais apolares que as demais própolis e apresentando uma excelente atividade antimicrobiana, verificada através da formação do halo de inibição contra *Staphylococcus aureus* e *Streptococcus mutans*. Foi encontrado um teor de flavonoides totais entre as amostras baianas variando de 4,36 a 26,44 mg g^{-1} de própolis. Moura (2000) salientou que a própolis da Bahia com maior atividade antimicrobiana apresentou o menor teor de flavonoides indicando que, possivelmente, a atividade antimicrobiana desse produto não se deve aos flavonoides. Cabral et al. (2012) estudou a própolis do tipo 6 obtida na Mata Atlântica da cidade de Entre Rios (BA) e encontrou teor de fenólicos de 1,48% e teor de flavonoide de 0,32%, considerado baixo em relação às outras própolis estudadas por eles. Apesar deste baixo teor, esta propolis também apresentou alta atividade microbiana.

Alencar et al. (2001) investigaram quimicamente própolis da região da Mata Atlântica, coletadas na cidade de Entre Rios (Bahia) e folhas de *Hyptis divaricata* Pohl ex Benth. (Lamiaceae), espécie mencionada como sua fonte botânica, mediante o uso das técnicas de cromatografia em camada delgada de alta eficiência - fase reversa (CCDAE-FR), cromatografia líquida de alta eficiência - fase reversa (CLAE-FR) e cromatografia em fase gasosa acoplada a espectrometria de massa (CG-EM). Eles concluíram que a composição dos compostos fenólicos encontrados na própolis na Mata Atlântica da Bahia possibilitou uma clara diferenciação dos outros grupos de própolis encontrados no Brasil. Além disso, a origem botânica desse tipo de própolis mostrou ser a espécie vegetal *H. divaricata*. Foi também avaliada, através de antibiograma, a própolis coletada em diferentes estações do ano, quanto à atividade antibacteriana contra *Staphylococcus aureus*, demonstrando que, qualitativamente, as amostras de própolis coletadas ao longo do ano demonstraram alta atividade antimicrobiana, o que poderia estar relacionado principalmente às benzofenonas isopreniladas. As benzofenonas são compostos fenólicos que apresentam alta atividade antimicrobiana, antitumoral e antioxidante, e estão entre os compostos químicos mais importantes identificados e isolados a partir de própolis encontradas em regiões tropicais, incluindo por exemplo, o estado da Bahia.

Comprovando mais uma vez o potencial antimicrobiano da própolis tipo 6 (Bahia), Duarte (2002) estudou o extrato etanólico da própolis bruta (EEP), que se comportou como um agente antimicrobiano bastante promissor, inibindo o crescimento de *Streptococcus mutans* em baixas concentrações, sendo bactericida para a maioria dos microrganismos testados, além de ser capaz de inibir cepas de coleção e também microrganismos isolados recentemente de pacientes. Este trabalho demonstrou que a própolis tipo 6 pode ter um efeito biológico *in vivo* na prevenção da placa dental cariogênica e cárie dental. Além disso, o estudo deixou claro que os compostos biologicamente ativos desta própolis têm características apolares, como apontado por outros autores, sendo encontrados tanto na fração hexana quanto na clorofórmica, mostrando-se importante o isolamento e a identificação destes compostos, assim como a análise de seus possíveis mecanismos de ação.

No estudo realizado por Castro et al. (2009a), foi verificado o perfil cromatográfico da própolis tipo 6 e foi possível identificar a benzofenona prenilada como um dos principais compostos. Castro et al. (2009b) também avaliaram a própolis do tipo 6 (Mata Atlântica da Bahia, 11°56'31" S e 38°05'04"W) em relação à concentração inibitória mínima (MIC) e à mínima concentração bactericida (MBC) dos extratos etanólicos e suas frações e subfrações orgânicas. No estudo, foram determinados valores de MIC que indicavam que a fração hexana exibia forte atividade antibacteriana contra *Staphylococcus aureus* e *Streptococcus mutans*, com concentrações tão baixas como 25 e 50 µg mL^{-1}, respectivamente, indicando a presença de um composto ativo nessa fração, possivelmente benzofenona, de acordo com a análise cromatográfica realizada. Valores semelhantes de MIC foram determinados por Daugsch (2007), que também avaliou a própolis tipo 6. Castro et al. (2009b) não detectaram flavonoides, nem os derivados de ácido cinâmico em amostras dessa própolis, o que foi confirmado através das análises por CLAE, CG-EM e HRGC-FID (cromatografia gasosa de alta eficiência-detector de ionização de chamas).

Outro estudo com a propolis tipo 6 da Bahia teve como objetivo avaliar seu efeito na mastite subclínica (Costa et al. 2011). Entretanto, nas concentrações testadas, não houve efeito positivo desta própolis como agente para o tratamento da patologia durante os dias avaliados (72 h). Já Simões (2008) avaliou a ação antimicrobiana de extratos comercias de própolis frente aos microrganismos presentes na saliva total e estimulada de humanos, entre os extratos comerciais utilizados, um era oriundo da Bahia (própolis do tipo 6). A análise dos resultados indicou que os extratos comerciais possuem ação antimicrobiana contra os patógenos bucais, assim como constatado em análise *in vitro*.

Righi (2008) estudou o teor de compostos fenólicos totais (%), flavonoides totais (%) e ceras (%) em própolis produzida na região de Cabo Verde (Bahia), verificando teores de 25,87 para compostos fenólicos, 3,15 para flavonoides e 22,59 para ceras. A porcentagem de compostos fenólicos e flavonoides obtida foi a terceira maior dentre as nove amostras avaliadas pelo autor, ficando atrás apenas da própolis produzida na região de Pirenópolis (Goiás) e em Maceió (Alagoas). Foi identificado também que os derivados de ácido cafeoilquínicos e fenilpropanóides prenilados e muitas flavonas glicosiladas estavam presentes nas amostras, como luteolina-o-rutinosídeo, chaftosídeo, crisoeriol-o-glicosídeo, isochaftosídeo e apigenina-di-o-glicosídeo, além de flavonóis glicosilados, como quercetina o-ramnosídeo e rutina, e, ainda, uma flavanona glicosilada (dimetoxi naringenina-di-C-glicosídeo).

A provável fonte vegetal comparada com a análise da composição química é o melhor indicador da origem botânica da própolis (Alencar et al. 2005). A análise polínica das amostras de própolis é uma técnica pouco utilizada na pesquisa, já que requer conhecimentos amplos de palinologia (Freitas 2002). A palinologia, que designa o estudo morfológico do grão de pólen, bem como sua dispersão e aplicações, teve início no século XVII, quando se desenvolveu a microscopia ótica, permitindo que estas partes diminutas das plantas pudessem ser observadas com precisão (Salgado-Labouriau 1973). Através da morfologia do grão de pólen é possível a identificação de táxons vegetais, permitindo a inferência sobre a composição da vegetação local e regional através de associações polínicas (Barth et al.1999; Barth & Luz 2009).

Existem diversas espécies vegetais para a retirada de resina pelas abelhas. Entretanto, poucas são as espécies identificadas até o momento; *Vernonanthura phosphorica* (Vell.) H.Rob. (assa-peixe; Asteraceae), *Schinus terebinthifolius* Raddi (aroeira; Anacardiaceae), *Baccharis* spp. (alecrim; Asteraceae) e *Eucaliputus* spp. (eucalipto; Myrtaceae) são alguns exemplos de espécies vegetais onde as abelhas buscam matéria-prima para a produção da própolis (Park et al. 2000). Barth & Luz (2009) em um estudo palinológico de amostras de própolis vermelha incluindo três amostras da Bahia coletadas em áreas de manguezais das cidades de Camamu, Mucuri e Prado identificaram a presença principalmente de resina de *Schinus*, conhecida popularmente como aroeira-pimenteira.

Dentre os oito documentos de patente, seis são de titularidade do Serviço Nacional de Aprendizagem Industrial (Bahia) e relatam o desenvolvimento de bebidas energéticas a base de própolis (PI011110000637, BR102013005726-6, BR102013005729-0, BR102013005723-1, BR102013005725-8 e BR102013005727-4); as outras duas patentes (PI0215610-5 e PI0215673-3) são de titularidade de Luciete Santos Ferreira ME, e referem-se a complementos alimentares e/ou energéticos que possuem própolis como um dos seus componentes ativos.

Considerações Finais

Neste levantamento, foram encontrados 17 trabalhos e oito documentos de patentes que relatam a produção de três tipos de própolis na Bahia, dentre as 13 encontradas para o Brasil. As própolis do grupos 6 e 13 possuem grande potencial para a indústria de alimentos e farmacêutica devido principalmente às suas características funcionais Os estudos estão concentrados na determinação da atividade antimicrobiana, obtendo resultados positivos e bem expressivos, principalmente para a própolis do tipo 6, caracterizada pelo baixo teor de flavonoides. A outra própolis de grande importância encontrada na Bahia é a do grupo 13 (vermelha), descoberta recentemente. Sua produção na Bahia ainda é reduzida, mas já existe incentivo do governo para acelerar a produção, principalmente por causa das atividades biológicas já comprovadas para este produto.

Referências

Adelman, J. 2005. *Própolis: variabilidade composicional, correlação com a flora e bioatividade antimicrobiana/oxidante*. Dissertação de Mestrado. Universidade do Paraná.

Alencar, S.M. 2002. *Estudo Fitoquímico da Origem Botânica da Própolis e Avaliação da Composição Química de Mel de* Apis melifera *africanizada de diferentes regiões do Brasil*. Tese de Doutorado. Universidade Estadual de Campinas.

Alencar, S.M.; Park, Y.K.; Aguiar, C.L. & Mariuzzo, D.M. 2001. Evidências fitoquímicas da própolis da região de Mata Atlântica do estado da Bahia. *Revista Baiana de Tecnologia* 16: 154–160.

Alencar, S.M.; Aguiar, C.L.; Paredes-Guzmán, J. & Park, Y.K. 2005. Composição química de *Baccharis dracunculifolia,* fonte botânica das própolis dos estados de São Paulo e Minas Gerais. *Ciência Rural* 25: 909–915.

Bankova, V.; Christov, R.; Kujumgiev, A.; Marcucci, M.C. & Popov, S. 1995. Chemical composition and antibacterial activity of Brazilian propolis. *Zeitschrift für Naturforschung* 50: 167–172.

Barbosa, M.H.; Zuffi, F.B.; Maruxo, H.B. & Jorge, L.L.R. 2009. Ação terapêutica da própolis em lesões cutâneas. *Acta Paulista de Enfermagem* 22: 318–22.

Barros, M.P.; Lemos, M.; Maistro, E.L.; Leite, M.F.; Sousa, J.P.; Bastos, J.K. & Andrade, S.F. 2008. Evaluation of antiulcer activity of the main phenolic acids found in Brazilian green propolis. *Journal of Ethnopharmacology* 120: 372–377.

Barth, O.M. 2006. Palynological analysis of geopropolis samples obtained from six species of Meliponinae in the Campus of the Universidad de Ribeirão Preto, USP, Brazil. *Apiacta* 41: 71–85.

Barth, O.M. & Luz, C.F.P. 2009. Palynological analysis of Brazilian red propolis samples. *Journal of Apicultural Research and Bee World* 48: 181–188.

Barth, O.M.; Dutra, V.M.L. & Justo, R.L. 1999. Análise polínica de algumas amostras de própolis do Brasil meridional. *Ciência Rural* 29: 663–667.

Bittencourt, F.O. 2008. *Desenvolvimento e Avaliação da Atividade Antimicrobiana Contra* Candida albicans *de Formulações Semi-sólidas Contendo Própolis Vermelha*. Dissertação de Mestrado. Universidade Tiradentes.

Brasil 2001. *Departamento de Inspeção de Produtos de Origem Animal. Regulamentos técnicos de identidade e qualidade do mel e produtos apícolas*. MAPA/DAS/ DIPOA/DNT, Brasília.

Breyer, E.U. 1982. *Abelhas e Saúde*. 2 ed. Uniporto Gráfica e Editora Ltda, Paraná.

Burdock, J.S. 1998. Review of the biological properties and toxicity of bee propolis. *Food and Chemical Toxicology* 36: 347–363.

Cabral, I.S.R.; Oldoni, T.L.C.; Alencar, S.M.A.; Rosalen, P.L. & Ikegaki, M. 2012. The correlation between the phenolic composition and biological activities of two varieties of Brazilian propolis (G6 and G12). *Brazilian Journal of Pharmaceutical Sciences* 48: 557–564.

Castro, M.L.; Nascimento, A.M.; Ikegaki, M.; Costa-Neto, C.M.; Alencar, S.M. de & Rosalen, P.L. 2009a. Identification of a bioactive compound isolated from Brazilian propolis type 6. *Bioorganic & Medicinal Chemistry* 17: 5332–5335.

Castro, M.L.; Vilela, W.R.; Zauli, R.C.; Ikegaki, M.; Rehder, V.L.G.; Foglio, M.A.; Alencar, S.M. de & Rosalen, P.L. 2009b. Bioassay guided purification of the antimicrobial fraction of a Brazilian propolis from Bahia state. *BMC Complementary and Alternative Medicine* 9: 25–30.

Costa, P.S.C. & Oliveira, J.S. 2005. *Manual Prático de Criação de Abelhas*. Editora Aprenda Fácil, Viçosa.

Costa, N.C.; Andrea, M.V.; Carvalho, G.J.L.; Almeida, A.M.L. & Ledo, C.A.S. 2011. Avaliação do efeito do extrato de própolis no tratamento da mastite subclínica em vacas de leite. *Magistra* 23: 257–261.

Daugsch, A. 2007. *A Própolis Vermelha do Nordeste do Brasil e suas Características Químicas e Biológicas*. Tese de Doutorado. Universidade Estadual de Campinas.

Daugsch, A.; Moraes, C.S.; Fort, P.; Pacheco, E.; Lima, I.B.; Abreu, J.A. & Park, Y.K. 2006. Própolis Vermelha e sua origem botânica. *Mensagem Doce*. 89. Disponível em http//:www.apacame.org.br; acesso em: 30 set. 2012.

Daugsch, A.; Moraes, C.S.; Fort, P. & Park, Y.K. 2008. Brazilian red propolis - chemical composition and botanical origin. *Evidence-Based Complementary and Alternative Medicine* 5: 435–441.

Duarte, S. 2002. *Estudo In Vitro do Efeito da Própolis da Bahia (Mata Atlântica) Sobre Estreptococos do Grupo* mutans *e na Atividade das Glucosiltransferases*. Dissertação de Mestrado. Faculdade de Odontologia de Piracicaba.

Freitas, A.S.; Vit, P. & Barth, O.M. 2012. Pollen profile of geopropolis samples collected by native bees (Meliponini) in South American countries. *Sociobiology* 59: 1–16.

Freitas, F.O. 2002. Uso da palinologia em amostras arqueológicas de própolis na reconstituição da vegetação histórica de uma região. *Boletim de Pesquisa e Desenvolvimento* 22: 1-17.

Koo, H.; Rosalen, P.L.; Cury, J.A.; Ambrosano, G.M.B.; Murata, R.M.; Yatsuda, R.; Ikegaki, M.; Alencar, S.M. & Park, Y.K. 2000. Effect of a new variety of *Apis mellifera* propolis on mutans streptococci. *Current Microbiology* 41: 192–196.

Korn, M.G.A.; Guida, M.A.B.; Barbosa, J.T.P.; Torres, E.A.; Fernandes, A.P.; Santos, J.C.C.; Dantas, K.G.F.; Nóbrega, J.A. 2013. Evaluation of sample preparation procedures for trace element determination in Brazilian propolis by inductively coupled plasma optical emission spectrometry and their discrimination according to geographic origin. *Food analytical methods* 6: 872–880.

Lengler, S. 2012. Própolis. Disponível em http:// www.brasilapicola.com.br/node/105; acesso em 3 set 2012.

Lima, G. 2008. *Estudo sobre Mel, Cera e Própolis*. ApexBrasil, Brasília.

Lima, M.G. 2006. *A Produção de Própolis no Brasil*. São Sebastião Editora e Gráfica, São João da Boa Vista.

Lustosa, S.R.; Galindo, A.B.; Nunes, L.C.C.; Randau, K.P. & Rolim Neto, P.J. 2008. Própolis: atualizações sobre a química e a farmacologia. *Revista Brasileira de Farmacognosia* 18: 447–454.

Menezes, H. 2005. Própolis: uma revisão dos recentes estudos de suas propriedades farmacológicas. *Arquivos do Instituto Biológico* 72: 405–411.

Moura, F.F. 2000. *Determinação das Propriedades Físico-químicas e Biológicas da Própolis Produzida por* Apis mellifera *na Região Nordeste do Brasil*. Dissertação de mestrado. Universidade Estadual de Campinas.

Moura, S.A.L.; Negri, G.; Salatino, A.; Lima, L.D.C.; Dourado, L.P.A.; Mendes, J.B.; Andrade, S.P.; Ferreira, M.A.N.D. & Cara, D.C. 2009. Aqueous extract of Brazilian green propolis: primary components, evaluation of inflammation and wound healing by using subcutaneous implanted sponges. *Evidence Based Complementary Alternative Medicine* 2011: 1–9.

Nascimento Junior, A.V. 2007. A produção de própolis no Brasil. In: Anais do IV Seminário de Própolis do Nordeste, ilhéus, 24–26 out. 2007, p. 36–43.

Nunes, L.C.C.; Galindo, A.B.; Deus, A.S.O.; Rufino, D.A.; Randau, K.P.; Xavier, H.S.; Citó, A.M.G.L. & Rolim Neto, P.J. 2009. Variabilidade sazonal dos constituintes da própolis vermelha em bioatividade em *Artermia salina*. *Revista Brasileira de Farmacognosia* 19: 524–529.

Oldoni, T.L.C.; Cabral, I.C.R.; D'Arcea, M.A.B.R.; Rosalen, P.L.; Ikegaki, M.; Nascimento, A.M. & Alencar, S.M. 2011. Isolation and analysis of bioactive isoflavonoids and chalcone from a new type of Brazilian propolis. *Separation and Purification Technology* 77: 208–213.

Park, Y.K. & Ikegaki, M. 1998. Preparation of water and ethanolic extracts of propolis and evaluation of the preparations. *Bioscience, Biotechnology, and Biochemistry* 62: 2230–2232.

Park, Y.K.; Ikegaki, M.; Abreu, J.A.S. & Alcici, N.M.F. 1988. Estudo da preparação dos extratos de própolis e suas aplicações. *Revista Ciência e Tecnologia de Alimentos* 18: 313–318.

Park, Y.K.; Ikegaki, M. & Alencar, S.M. 2000. Classificação das própolis brasileira a partir de suas características físico-químicas e propriedades biológicas. *Mensagem Doce* 58. Disponível em http//:www.apacame.org.br; acesso em 3 set. 2012.

Paulino, F.D.G. 2004. Produtos da colmeia. In: D.C. Souza, (ed.), *Apicultura: manual do agente de desenvolvimento rural*. SEBRAE, Brasília.

Pereira, A.S.; Seixas, F.R.M.S. & Neto, F.R.A. 2002. Própolis: 100 anos de pesquisa e suas respectivas futuras. *Química Nova* 25: 321–326.

Pontin, K.; Silva Filho, A.A.; Santos, F.F.; Silva, M.L.; Cunha, W.R.; Nanayakkara, N.P.; Bastos, J.K. & Albuquerque, S. 2008. *In vitro* and *in vivo* antileishmanial activities of a Brazilian green propolis extract. *Parasitology Research* 103: 487–492.

Righi, A.A. 2008. *Perfil Químico de Amostras de Propólis Brasileiras*. Dissertação de Mestrado. Universidade São Paulo.

Salgado-Labouriau, M.L. 1973. Contribuição à Palinologia dos Cerrados. Academia Brasileira de Ciências, Rio de Janeiro.

SECOM (Secretaria de Comunixação Social) 2011. EBDA estimula produção de própolis vermelha no sul do estado. Notícias – Agricultura, 11 ago. 2011. Disponível em http://www.comunicacao.ba.gov.br/noticias/2011/08/11/ebda-estimula-producao-de-propolis-vermelha-no-sul-do-estado; acesso em: 1 out. 2012.

SEBRAE (Serviço Brasileiro de Apoio às Micro e Pequenas empresas) 2010. *Exportação Brasileira de Cera de Abelha (Própolis)*. Disponível em http://www.sebrae.com.br/setor/apicultura/sobre/apicultura/mercado/exportacoes/Exportacoes%20Julho.pdf; acesso em 14 ago. 2012.

Simões, C.C.; Araújo, D.B. & Araújo, R.P.C. 2008. Estudo *in vitro* e *ex vivo* da ação de diferentes concentrações de extratos de própolis frente aos microrganismos presentes na saliva de humanos. *Revista Brasileira de Farmacognosia* 18: 84–89.

Sousa, J.P.B.; Furtado, N.A.J.C.; Jorge, R.; Soares, A.E.E. & Bastos, J.K. 2007. Perfis físico-químico e cromatográfico de amostras de própolis produzidas nas microrregiões de Franca (SP) e Passos (MG), Brasil. *Revista Brasileira de Farmacognosia* 17: 85–93.

Souza, A.P.B.; Passos, J.P.G.; Padilha, A.F.F.; Albuquerque Júnior, R.L.C. & Cardoso, J.C. 2009. Efetividade da *Delphinum staphysagria* 6cH e 30cH em ensaios biológicos para cicatrização. *Brazilian Homeopathic Journal* 11: 13–14.

Souza, H.R.; Pando, A.M.S.C.; Cruz-Barros, M.A.V. & Albuquerque, P.M.C. 2012. Espectro polínico da própolis da abelha sem ferrão Tubi – *Scaptotrigona* aff. *postica* Latreille, 1807 (Hymenoptera: Apidae: Meliponini) em Barra do Corda - MA - Brasil. In: 19ª Reunião Anual do Instituto de Botânica, São Paulo, 2012.

Teixeira, E.W.; Message, D.; Meira, R.M.A. & Salatino, A. 2003. Indicadores da origem botânica da própolis: importância e perspectivas. *Boletim de Indústria Animal* 60: 83–106.

Teixeira, E.W.; Negri, G.; Meira, R.M.; Message, D. & Salatino, A. 2005. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. *Evidence-based Complementary and Alternative Medicine* 2: 85–92.

Capítulo 5. Artigo

6.0 CAPÍTULO 5. Determination of parameters for the supercritical extraction of antioxidant compounds from green propolis using carbon dioxide and ethanol as co-solvent

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RESEARCH ARTICLE

Determination of Parameters for the Supercritical Extraction of Antioxidant Compounds from Green Propolis Using Carbon Dioxide and Ethanol as Co-Solvent

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Abstract

The aim of this study was to determine the best processing conditions to extract Brazilian green propolis using a supercritical extraction technology. For this purpose, the influence of different parameters was evaluated such as S/F (solvent mass in relation to solute mass), percentage of co-solvent (1 and 2% ethanol), temperature (40 and 50°C) and pressure (250, 350 and 400 bar) using supercritical carbon dioxide. The Global Yield Isotherms (GYIs) were obtained through the evaluation of the yield, and the chemical composition of the extracts was also obtained in relation to the total phenolic compounds, flavonoids, antioxidant activity and 3,5-diprenyl-4-hydroxicinnamic acid (Artepillin C) and acid 4-hydroxycinnamic (p-coumaric acid). The best results were identified at 50°C, 350 bar, 1% ethanol (co-solvent) and S/F of 110. These conditions, a content of 8.93±0.01 and 0.40±0.05 g/100 g of Artepillin C and p-coumaric acid, respectively, were identified indicating the efficiency of the extraction process. Despite of low yield of the process, the extracts obtained had high contents of relevant compounds, proving the viability of the process to obtain green propolis extracts with important biological applications due to the extracts composition.

Introduction

Propolis is a resinous material collected by bees from sprouts and barks of different plants and trees. It is originally used as a substance of defence for the hives [1–3]. Propolis has a pleasant

Competing Interests: The authors have declared that no competing interests exist.

aromatic odour and its colour varies from green-yellow, dark brown to red, depending especially on the vegetable source, season and its geographical origin. *Baccharis dracunculifolia* DC (Asteraceae) is a native plant from Brazil and it is considered the most important botanical source of green propolis, predominant in the Brazilian southeast [4]. Different studies have demonstrated the biological properties of propolis extracts such as its anti-oxidant action [5–6], antimicrobial action [7–9], anti-inflammatory action [10–11], anti-parasite action [12–13], immune-modulatory action [14–15], anti-tumor action [16–19], antiviral action [20–21] and others [22–23]. These biological activities are especially attributed to the phenolic acids, flavonoids, terpenes and sesquiterpenes present in the propolis [24–26].

The physiological and biochemical mechanisms responsible for the biological effects of propolis are still to be determined. The majority of the therapeutic effects, however, were suggested through an association between its anti-microbial actions and the capacity to sequestrate free radicals [27–28]. Furthermore, the anti-oxidant and anti-inflammatory effects have been extensively attributed to its high flavonoid content [29]. One of the main phenolic acids presents in samples of green propolis is the 3,5-diprenyl-4-hydroxicinnamic acid (HPPC), also known as Artepillin C. This compound is particularly relevant for the pharmaceutic industry, considering its preventive and anti-tumoural effects *in vitro* and *in vivo*, identified in some studies [2, 30–34].

Considering the different types of processes used around the world to obtain propolis extracts, ethanol is the first choice of solvent, especially due to the affinity of its chemical characteristics with the matrix. Other solvents such as ethylic ether, water, methanol and chloroform can also be used for the extraction of specific classes of propolis constituents [35–36]. The extracts can be obtained through conventional techniques such as extraction by Soxhlet, maceration, or alternative methods, such as extraction with supercritical fluid [24–25,37–39]. Supercritical fluid extraction (SFE) shows very desirable characteristics, considering its high flexibility and it can adjust the solvent power and process selectivity. Besides, the high quality of the product obtained, when compared to the conventional methods, shows more advantages due to low use of polluting organic solvents. In the last decades, SFE has been widely used for the extraction of scents, fragrances, as well as active constituents, especially from vegetable matrices [40]. Supercritical fluids have low viscosity as gas, high density as liquids, and an intermediate level of diffusion somewhere between gases and liquids, varying with its density [41–43]. Carbon dioxide is the most widely used solvent due to its low cost, easy available in high purity levels, non-toxic, non-flammable and non-explosive. Another advantage is that the carbon dioxide is a gas at room temperature and pressure, also easily removed after the extraction process [44].

Analysing the great relevance given to the SFE technology as an alternative extractive process, and the importance and promising biological effects of the Brazilian green propolis, the aim of this work was to define the best conditions to the process. This includes establishing the temperature, pressure and co-solvent concentration necessary for the extraction of green propolis, using carbon dioxide as a supercritical fluid. In this sense, the global curves of extraction, yield and global extraction isothermals were defined by the compounds studied, as well as the characterization of the extracts obtained, related to some components: 3,5-diprenyl-4-hydroxicinnamic acid (Artepillin C) and acid 4-hydroxycinnamic (p-coumaric acid).

Materials and Methods

Obtainment and processing propolis

Approximately two kilos of green propolis were donated by the company Apis Nativa Produtos Naturais (Prodapys–Santa Catarina–Brazil), originated from the south of the Paraná state,

Brazil. The sample of green propolis was grinded in a mill (Cadence–Brazil) and then sieved, aiming to obtain an adequate particle size (diameter 52–92 μm) to increase the surface area and homogeneity of the starting material in the extraction bed. Small quantities of propolis (100 g) were kept at -10°C, in bottles protected with aluminium foil in inert atmospheric conditions (N_2), in order to avoid degradation of the material.

Materials and reagents

Ethanol (HPLC degree) and acetic acid (HPLC degree) were obtained from Merck Co. (Darmstadt, Germany) and methanol (PA) from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A cellulose ester membrane filter of 0.45 μm (SLCR025NS, Millipore Co., Bedford, Massachusetts, USA) was used. The carbon dioxide (CO_2) used in the extraction had 99.9% purity (White Martins Industrials Gases–São Paulo, Brazil). The standard 3,5-diprenyl-4-hydroxicinnamic acid (Artepillin C–cas number 72944-19-5) was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and the acid 4-hydroxycinnamic (p-coumaric acid–cas number 501-98-4), 1,1-diphenil-2-picrilhidrazil (DPPH), Acid Gallic (cas number 149-91-7) and Quercetin (cas number 117-39-5) were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Characterization of raw material

The determinations of humidity, protein and total ash contents were made according to the official methods of Association of Official Agricultural Chemists (AOAC) [45]. The total lipids were extracted and quantified through the cold extraction method described by Bligh & Dyer [46]. The determination of the mineral content was made in a digital flame photometer (DM-62, DIGIMED, São Paulo—Brazil) and the fibre content was obtained through the automatic fibre analyser (A-220, ANKON, New York–USA) [47]. The quantification of the water activity took place using a decagon LabMaster (Novasina, Lachen–Switzerland), with electrolytic cell CM-2. The analyses were performed in triplicate. The Scanning electron microscopy (SEM) was performed in a scanning electron microscope JEOL JSM-6390LV (USA). After drying in an oven (105°C/45 min), the sample of crushed propolis was fixed manually using a tweezer (PELCO Tweezers) of aluminum metal surfaces covered with carbon double-sided tape, called stubs. Because of the need for interaction of the electron beam with the sample, it was performed by coating deposition of metallic gold ions (sputtering). The sample was metalized in gold in a "Sputter oater" from Balzers, model SCD 50 (20nm). Then the stubs containing the metallic samples were stored in plastic boxes (storage boxes), duly sealed with parafilm (PARAFILM M) to prevent moisture absorption. After 24 hours of rest, the samples were analyzed at different magnifications (Voltage 12 kV, Working Distance 12 mm, Spot size 44, Vacuum Mode HV).

Extraction Procedure

The equipment used to obtain the extracts of green propolis was a pilot unity called SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc.), composed by a high pressure bomb (capacity up to 10,000 psi), extraction cell (capacity 100ml), oven (with a preheater), static/dynamics valve and restrictor valve, flow meter and CO_2 cylinder (S1 Fig). A CO_2 cylinder with fishing tube was used to ensure that only CO_2 in liquid state was used in the system, a requirement demanded by SFT-110. The cell was assembled in the oven and kept at the preselected temperature. The temperature of the restrictor valve was adjusted to 80°C for all extraction processes and 50°C for cleaning. The extract was collected in a 50 ml glass vial, immersed in ice at room temperature and the total of CO_2 was measured using a flow totalizer (ITRÓN, ACD G1.0, Argentina). The output of CO_2 in the system was of 6.0 g/min in all experiments.

The extraction processes were described graphically, by the curves obtained through the analyses of the extracts yield over time, using extraction kinetics and the isotherms obtained. The definition of the best extraction conditions consisted of three phases, described below. All experiments were performed in triplicates.

Phase 1 – Determination of the extraction global curve and S/F (CO_2 mass/propolis mass). The pilot kinetics of extraction was performed in the mildest of temperature and pressure (100 bar and 40°C) using 7.5 g of sample and CO_2 flow of 6 g/min. At pre-determined periods, the collection bottles used for extraction were substituted for clean ones previously weighted, until no more extraction was being yielded. The extract mass contained in each bottle was measured using an analytical scale (SHIMADZU—São Paulo, Brazil), and thereafter the accumulated yield of the extract versus the corresponding accumulated S/F was represented in a graph, in order to observe the system behaviour. The S/F was calculated as the rate between the total mass of the solute (considering the volume of CO_2 in the system and its density) and the mass of feed on a damp base (7.5 g) [48]. The experiment lasted until the bed was considered exhausted, and 13 experiments in each assay were obtained. The total mass of the extract was determined as the addition of the extract obtained during extraction and the extract recovered in the cleaning process. The cleaning process consisted in recovering the extract inside the tube line that conducts it out of the extraction recipient with CO_2 and ethanol. The glass bottles (used in the processes, extraction and cleaning) were purged with N_2 and stored at -10°C until analysis. The global yield was calculated by the rate between the extract mass (Mextract) obtained and the propolis mass (Mpropolis) used to make the bed. The S/F adopted for the subsequent extractions was determined by the analysis of the obtained graph. For each collection bottle, in addition to order the yield of extraction, the content of total phenols and anti-oxidant activity were also determined.

Phase 2 – Determination of co-solvent percentage. In this phase, extractions were performed in triplicates, using 1% and 2% ethanol in relation to the CO_2 mass used in the process. These experiments were performed in conditions of 50°C, 250 bar and flow of CO_2 at 6 g/min [2,24–25,28,38,49]. The ethanol was homogenised with the sample and placed in the extraction cell, together with wool and glass pearls to fill the cell (S2 Fig). The adopted S/F was that obtained from the pilot kinetic at the previous phase (S/F = 110). The choice of percentage for the co-solvent adopted for the extraction of active compounds from propolis was determined through the characterization of the extracts (total phenols, anti-oxidant activity and flavonoids) and the quantification of p-coumaric acid and Artepillin C.

Phase 3 – Global yield isotherms (GYIs). The GYIs were elaborated aimed at identifying the ideal conditions of pressure and temperature for extraction of the studied matrix. They were determined at 40 and 50° C and 250, 350 and 400 bar [48,50]. The extraction conditions were those determined in previous phases (S/F = 110, 1% co-solvent and total extraction time of 2 h 30 min). The extraction cell consisted of 7.5 g green propolis (homogenised in co-solvent), wool and glass pearls packed together to avoid the preferred paths of CO_2 and totally fill the bed. The supercritical extraction process occurred using the desired pressure in the system, keeping the system pressurized for 30 minutes, followed by opening the valve and collecting the extract for 2 hours (S/F = 110) [48] (S3 Fig). The extracts of each assay were characterized by analysing the total phenol content, total flavonoids, anti-oxidant activity (IC_{50}), p-coumaric acid and Artepillin C.

Chromatographic Analysis

Solutions of 10 mg/ml of propolis extracts obtained in the different conditions of the process were prepared and dissolved in ethanol and placed in ultrasound bath (Sanders, SONICLEAN

6 –Minas Gerais, Brazil) for 30 minutes (Electronic timer microprocessor–Temperature 35°C electronically controlled and Ultrasound frequency 40 kHz). The exposure to the ultrasound system only started after reaching 35°C. The samples were filtered in a cellulose ester membrane filter 0.45 μm (Millipore) for subsequent injection in the high performance liquid chromatograph (HPLC).

The chromatographic experiments were performed with the system HPLC EZChrom Elite, consisting of a VRW HITACHI L-2130 pump, equipped with an automatic injector and diode arrangement detector (DAD) VRW HITACHI L-2455 and oven VRW HITACHI L-2300. The chromatographic separation was based in the method proposed by Daugsch [51], adapted. The column LiChroCART Purospher StaR RP18-e (75 mm x 4 mm i.d.) (3 μm) (Merck, Darmastad, Germany) was used together with a pre-column LiChroCART 4–4 LiChrospher 100RP18 (5 μm) from Merck.

The conditions for analysis were performed with an elution gradient with a mobile phase of acetic acid 5% (aqueous phase) and methanol (organic phase) in different proportions and with total analysis time of 70 minutes (0 min—80:20; 10 min 70:30; 15 min—60:40; 30 min—50:50; 45 min—40:60; 60 min—30:70; 65 min—0:100; 70 min—80:20). The volume of injection was of 10 μL. The equipment was operated at room temperature (25±2°C). The reading of the diode arrangement detector was in the range of 200 to 400 nm and the chromatographic acquisition was defined at 290 nm. The identification of the compounds was performed through the comparison of time of retention and ultraviolet spectrum between the samples and the controls (standard) (S4 and S5 Figs). In the Fig 1 are illustrated the chromatograms obtained to the standards analysed. Aiming at ensuring the reliability of the results obtained, a validation took place according to the National Health Surveillance Agency (ANVISA) [52] and National Institute of Metrology, Standardization and Industrial Quality (INMETRO) [53] methodologies. This was done in accordance to the parameters of selectivity, linearity, precision, accuracy, detection limits and quantification limits.

Determination of the Total Phenolic Compounds

The total phenolic content was determined using the Folin Ciocalteau reagent [54–55]. The reaction was prepared with a 0.5 ml aliquot of propolis extract (dissolved in ethanol aimed at obtaining a concentration of 200 μg/ml), 2.5 ml aqueous solution of Folin-Ciocalteau 10% and 2.0 ml sodium carbonate at 7.5%. The mixture was introduced in a thermo-regulated bath at 50°C for 5 minutes; afterwards, the absorbance was measured in a spectrophotometer LAMBDA 25 UV/vis Systems (PerkinElmer, Washington—USA) at 765 nm.

The quantity of total phenolic was expressed as Gallic acid equivalents (g of Gallic acid (GAE) in 100 g) through a calibration curve ($y = 0.0073x - 0.066$ $R^2 = 0.9995$) using known solutions to Gallic acid standard in the same conditions ($\lambda = 765$ nm). The Folin Ciocalteu method is associated to the appearance of a blue colouring due to the oxidation of phenols in basic medium [56].

Determination of Flavonoid content

The determination of flavonoid content was performed through the reading in a spectrophotometer (LAMBDA 25 UV/Vis Systems—PerkinElmer USA) at 415 nm, using aluminium chloride at 2% in methanol [57] in a 1:1 solution (extract:aluminium chloride). The same procedure was performed using known solutions of quercetin standard to elaborate a standard curve ($y = 0.0276x - 0.0256$ $R^2 = 0.9996$). The quantity of total flavonoids was expressed as quercetin equivalents (g of quercetin (GQ) in 100 g).

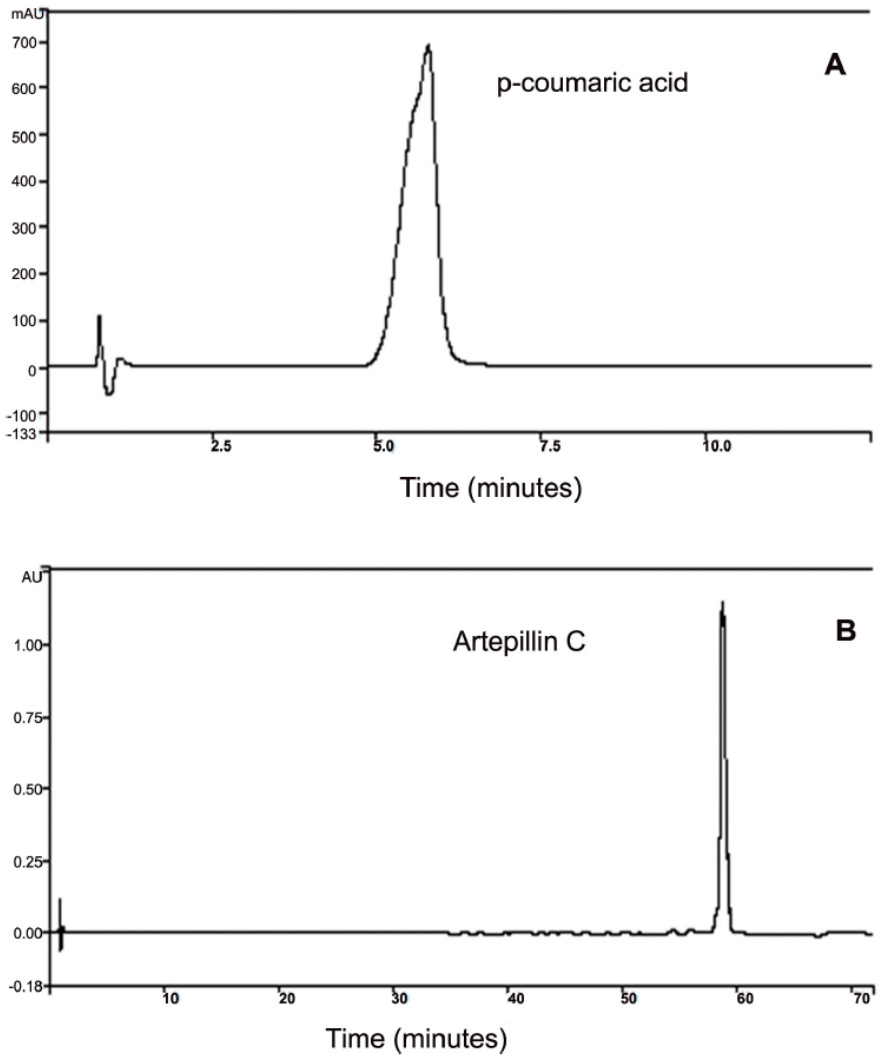

Fig 1. Chromatograms obtained to the p-coumaric acid and Artepillin C standards at 290 nm by HPLC.

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Determination of Anti-oxidant Activity in vitro (1,1-diphenil-2-picrilhidrazil–DPPH)

The anti-oxidant activity in vitro of propolis extracts obtained in different conditions was evaluated using the reactive 1,1-diphenil-2-picrilhidrazil (also known as the capacity to sequestrate the radical DPPH) [58–59]. Five dilutions of the extracts were prepared (20 to 400 μg/ml) in triplicates. An aliquot of 1 ml of each extract dilution was transferred to assay tubes with 3.0 ml of the ethanoic solution (95%) of the radical DPPH (0.004%). After 30 min incubation in the dark and at room temperature, the reduction of the free radical DPPH was measured through the reading of absorbance in 517 nm (calibration curve $y = 0.1897x - 4.5$ $R^2 = 0.9955$) (spectrophotometer LAMBDA 25 UV/Vis Systems–PerkinElmer, Washington—USA).

The same procedure was adopted with ethanol instead of the sample, considered white. The capacity to sequestrate free radicals was expressed as the percentage of oxidation inhibition in

the radical and calculated according to Equation 1. The IC_{50} value (necessary concentration of the extract to sequestrate 50% of DPPH radical) was calculated through the line equation based on the concentrations of extracts and its respective percentages of radical DPPH sequestration.

$$\%\ sequestration = 100 - \frac{\text{final absorvance of sample x } 100}{\text{white absorvance}} \quad (1)$$

Statistical Analysis

The results found were evaluated using analyse of variance ANOVA (one-way) and the Tukey Test, in order to identify whether the alterations in the parameters evaluated were significant at 95% confidence.

Results and Discussion

Characterization of raw propolis

The green propolis evaluated in this study presented a physical-chemical profile of 7.13±0.12% for humidity, 3.05±0.03% for total ash, 9.98±0.83% for proteins, 45.75±1.71% for lipids and 20.89±1.39% for fibres. There are very few works reporting the centesimal characterization of propolis. However, the values obtained in this study are similar to those found in the literature for other samples of propolis, which presented variations of 3.4–7.7% for humidity, 1.6–4.4% for total ash and 6.5–32.3% for lipids [25,60–65]. The variations found for the centesimal profile could be due to the type of propolis studied, geographical region, environmental conditions, and even to the collection period [64,66–68]. It should be highlighted that the centesimal composition of the studied sample is in agreement with the specifications established by the Brazilian legislation [69], as well as the legislations of other countries (Argentina and Switzerland). Regarding the water activity (Aw), the sample showed a value of 0.704±0.008, considered as a product with intermediate Aw. The Aw is considered a parameter totally linked to the humidity of the product, which allows the determination of its capacity for conservation, microbial propagation and occurrence of chemical reactions [70]. From the ash obtained, a mineral composition was performed in relation to the contents of sodium, potassium, lithium and calcium. The values found were 3.00±0.01, 331.70±5.81, 1.80±0.01 and 9.00±0.01 mg/Kg, respectively. The main micro-elements present in propolis samples are aluminium, calcium, strontium, potassium, iron, copper and manganese [1]. The determination of the percentage of micro and macro nutrients in propolis samples have a great importance to the nutritious knowledge and calorific value of diet in different animals, such as fish, chicken and milk cows [65,71–72].

On Fig 2, there are the micrographs of the green propolis particles used as an extraction material. The majority of the particles are presented in isolated form (Fig 2A), however with a tendency to form big agglomerates (Fig 2C). The same profile was presented in the work of Tylkowski et al., [73] for samples of propolis originated from Bulgaria. In all images, it is possible to observe rugged surfaces covered by layers of wax and extractives, besides revealing the presence of vegetal constituents, probably non-glandular trichomes and/or glandular and resinous substances from vegetative apexes of *Baccharis dracunculifolia* (Fig 2B, 2C and 2D) [4,74–76].

Determination of the extraction global curve and S/F

Initially, a pilot kinetic of extraction (kinetic curve) was determined, aimed at determining the quantity of solvent (CO_2) and consequently the time of extraction necessary to reach the

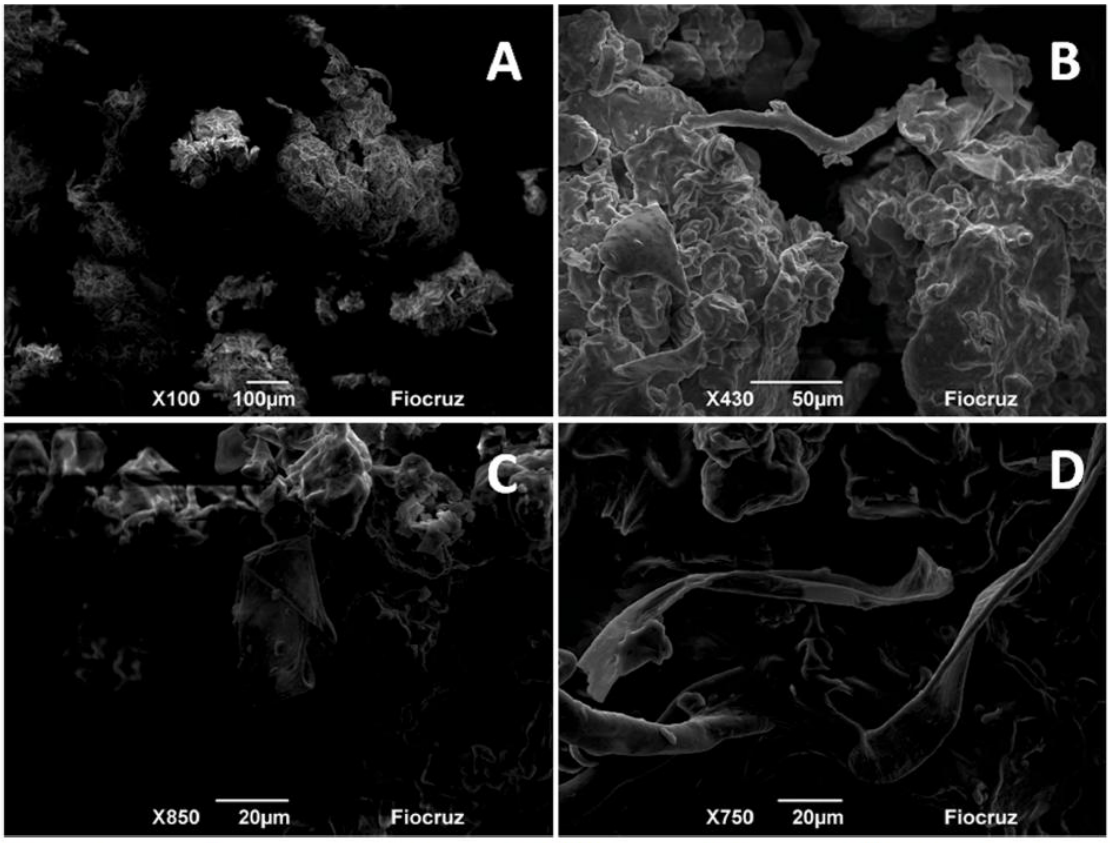

Fig 2. Images obtained by Scanning electron microscopy (SEM) for the sample of green propolis (Zoom of 100—A, 430—B, 800—C and 750—D times).

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diffusional period and to ensure that the global yield assays had results, which were close to the real exhaustion of the extraction bed, maximizing the quantity of extract obtained. For that purpose, milder temperature and pressure than those studied were used (40°C; 100 bar).

In preliminary assays, a 15g mass of sample was used for the extraction process (40°C, 100 bar, 6.0 g/min CO_2 flux, 15 g propolis) and subsequent obtainment of pilot kinetic curve. However, during the experiment, it was needed to reduce the quantity of samples, considering that after 327 minutes of extraction, there still was a great quantity of extract getting out, which demanded a large expenditure of energy and time to finalize the process. According to Meireles [50], considering that the exhaustive extraction process can take hours of experimental work, the sample mass reduction can influence positively to the lower energy expenditure. The reduction of the sample mass will not influence the selection to determine the S/F and the selection of the best conditions of temperature and pressure

As mentioned in the methodology, a mass of 7.5 g of green propolis was adopted to determine the extraction kinetic curve. The accumulated yield (extract mass) obtained and its corresponding S/F, as well as the results for the accumulated yield for total phenolic compounds and antioxidant activity (%) are presented in Table 1. On Fig 3, the kinetic curve obtained by the quantity (mass) of extract (values not accumulated) versus time of extraction is presented (S1 Table). On Fig 4, the extraction global curves for yield, phenolic compounds and antioxidant activity versus S/F value are presented. The same profile of extraction found by the propolis in

Table 1. Determination of the pilot kinetic of extraction for green propolis with accumulated extract mass yield (%), total phenolic content yield (%) and antioxidant activity (%) by each experiment obtained (mean ± standard deviation) (Conditions: 40°C, 100 bar, CO_2 flux of 6 g/min and 7.5 g green propolis).

Number of experiment	Time (min)	Volume of CO_2 (m^3)	S/F	Mass of Extract (Accumulated yield %)	Total Phenolic (Accumulated yield %)	Antioxidant activity (Accumulated yield %)
1	8.68	0.030	7.28	0.8346±0.0650	11.56±0.43	16.39±0.81
2	18.39	0.030	14.56	1.2073±0.1329	19.97±0.72	26.01±0.91
3	29.43	0.030	21.84	1.4086±0.1404	23.97±0.60	31.55±0.83
4	45.68	0.045	32.76	1.6146±0.0678	29.11±0.41	37.99±0.60
5	58.68	0.045	43.68	1.7206±0.0329	32.27±2.05	41.43±0.49
6	78.50	0.060	58.25	1.8926±0.0782	37.35±2.19	47.51±0.47
7	100.50	0.060	72.81	2.0573±0.1989	43.47±0.77	53.89±0.42
8	121.50	0.060	87.37	2.1933±0.2564	49.52±1.31	59.46±0.76
9	150.50	0.090	109.22	2.3960±0.3661	56.71±2.02	67.53±1.30
10	187.50	0.090	131.06	2.5373±0.4091	61.27±2.35	73.09±1.71
11	224.50	0.090	152.91	2.6846±0.4440	66.76±2.57	79.04±1.94
12	252.50	0.090	174.75	2.8286±0.5383	73.49±3.18	85.68±2.56
13	288.50	0.090	196.60	2.9720±0.5977	79.67±3.65	92.80±3.01

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this study was also identified by other natural samples using supercritical CO_2 such as for obtaining tagitinine C from *Tithonia diversifolia* [77], peach (*Prunus persica*) [78] and for cocoa beans [79].

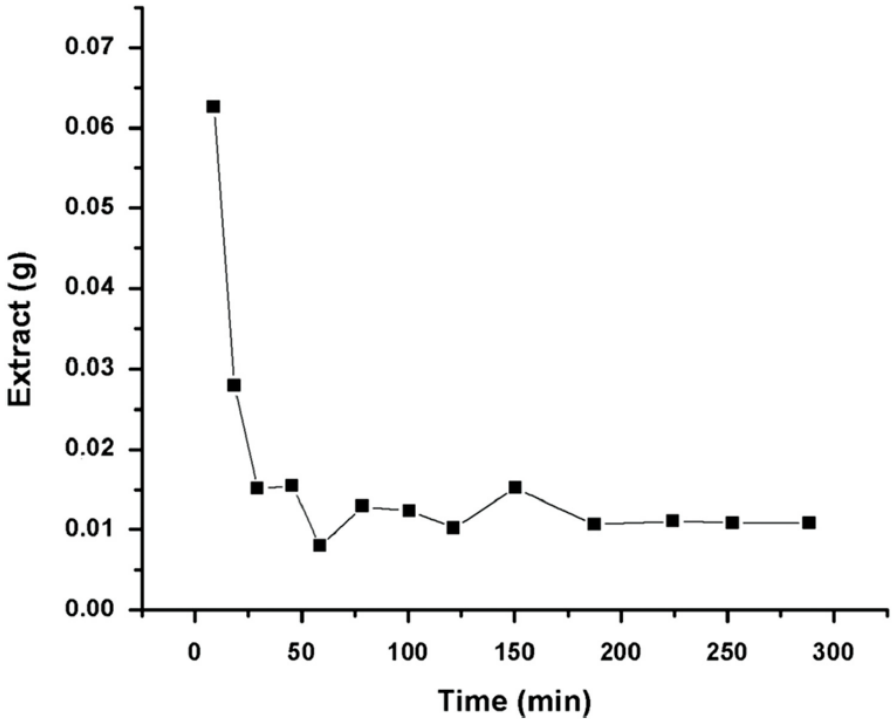

Fig 3. Profile of extraction for green propolis in relation to the extract mass obtained at different times for obtaining the pilot kinetic (Conditions: 40°C, 100 bar, CO_2 flux of 6 g/min and 7.5 g of green propolis).

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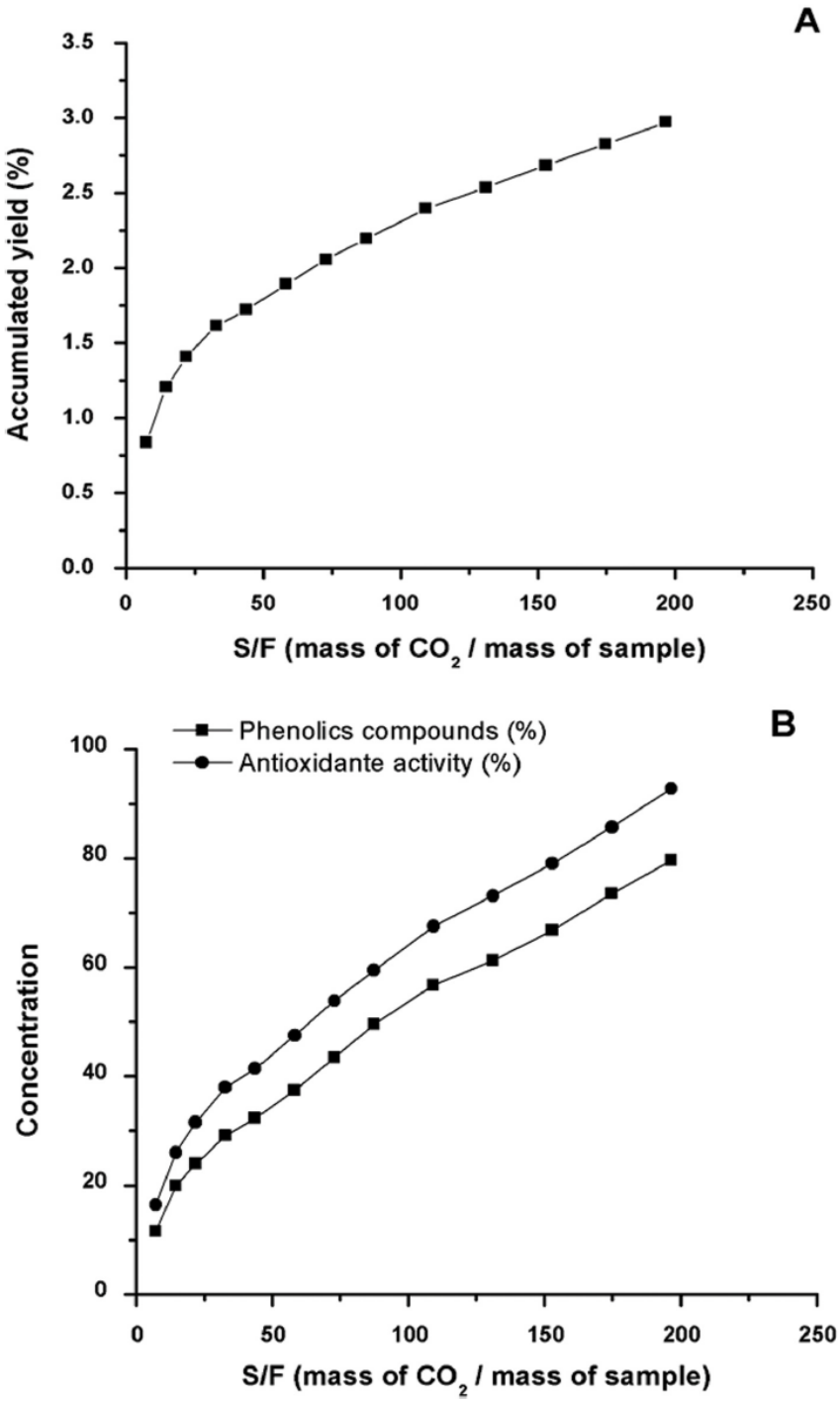

Fig 4. Global Curves of Extract for: (A) yield in mass of accumulated extract; (B) yield of the phenolic compound content and of the antioxidant activity (accumulated values) (Conditions: 40°C, 100 bar, CO_2 flux of 6.0 g/min and 7.5 g of green propolis).

doi:10.1371/journal.pone.0134489.g004

After analysing the behaviour of extraction using supercritical CO_2 under the conditions determined for the propolis and obtaining the global curves of extraction, the value of 110 was identified as the preferred S/F (Fig 3). At this point, a yield of 0.129±0.01% was determined for the quantity of extract obtained. A low yield was already expected in the process of propolis extraction by SFE, as it has been reported by other studies [24,38,80–81]. Furthermore, as described by Catchpole et al. [24], propolis is not very soluble in supercritical CO_2, but it is much more soluble in a mixture of CO_2 + ethanol. For that reason, the lack of a co-solvent with polar characteristics can implicate in a low yield in this phase of the process.

Different authors reported as the best extraction S/F the one corresponding to the global curve phase (of yield in extract mass) between the end of the increasing rate of extraction and the start of the decreasing rate [48,82–83]. The extraction curve of a natural matrix using supercritical fluids is not a linear function of time [82,84]. The curve presented in Fig 4A represents a pattern to extract natural products, also identified for the sample of black pepper (*Piper nigrum*, L.) by Ferreira and Meireles [85], for the sample of propolis by Biscaia and Ferreira [25] and for the sample of annatto (*Bixa orellana* L.) by Albuquerque and Meireles [48]. An initial linear part was identified (constant extraction rate = CER), followed by a decreasing rate and ending at a near zero extraction rate. The period determined as CER is that in which exists the presence of an easily accessible solute on the surface of the matrix particles. The resistance to the mass transference is primarily on the external region of the particle [25,82,85–87]. In this situation, the process of mass transference is controlled by convection (solvent flow). In standard models of an extraction global curve for supercritical fluids there are three typical phases: (i) constant extraction rate (CER); (ii) falling extraction rate (FER); and (iii) diffusional region (DF). In general, 50 to 90% of the total extractable material is obtained in the constant region of extraction rate, and the optimization process should be focused on this region [82]. Albuquerque and Meireles [48] also performed preliminary kinetic assays to select the parameters of the process which are more adequate to determine the GYIs, when they analysed annatto (*Bixa orellana* L.) and identified 35 as the best value of S/F for the studied sample.

Since one of the objectives of this study was to define the conditions of the process for a better and greater extraction of the propolis bioactive compounds such as the antioxidant compounds (polyphenols); the global extraction curves for yield in total phenols and antioxidant activity (Fig 4B) were determined. This was aimed at verifying the content of the compounds obtained according to the S/F employed, also considering the time of extraction. It is possible to identify on the Fig 4B that, where the S/F value is 110, there is approximately 60% of the extractable phenolic compounds (in the conditions used). It was observed that by increasing the S/F to 150, the yield of these compounds increases 8%, what may not justify the time and solvent (CO_2) expenditure and other costs in the process (if chosen the S/F higher than 110). In addition, the S/F of 110 was determined in the CER, which as reported in different studies, it is represented by a constant region extraction, and further where the and further where the solute is easily accessible on the surface of the matrix particles, facilitating the extraction process [82,84,87]. On Fig 5, the profile of extraction in relation to the content of total phenolic compounds and antioxidant activity is presented (not accumulated values).

Determination of co-solvent concentration

After the kinetic assays were performed, the experiments were developed to determine the percentage of co-solvent (m/m) to compound the extraction system and afterwards, it selects the process best parameters to determine the GYIs. The polyphenols are the main components of interest in propolis, which has several hydroxyl groups. They are hydrophilic molecules, due to this characteristic, methanol, ethanol and water have been used as solvents in its extraction.

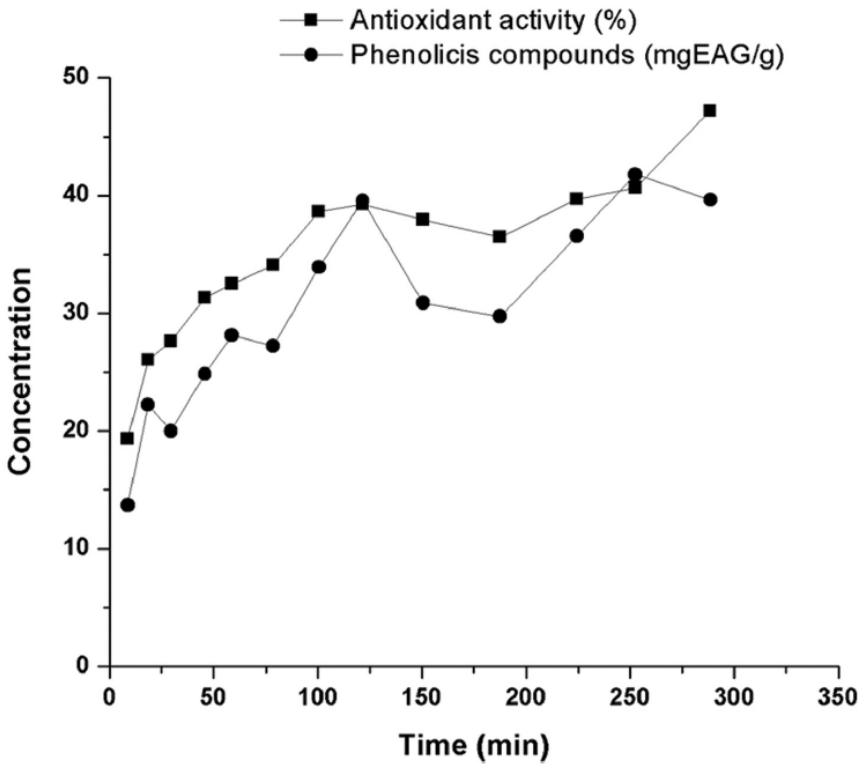

Fig 5. Profile of extraction of green propolis in relation to the content of phenolic compounds and antioxidant activity obtained at different times of extraction to determine the pilot kinetic curve (Conditions: 40°C, 100 bar, CO_2 flux of 6.0 g/min and 7.5 g green propolis).

doi:10.1371/journal.pone.0134489.g005

Solvents such as ethyl acetate, have also been used, and also aqueous mixtures of methanol, ethanol or acetone are often the choice of solvents for the recuperation of a wide range of phenols from several types of samples [88]. The ethanol was selected as a co-solvent due to the different studies showing that the presence of this component intensifies the extraction of the compounds of a less lipophilic nature in propolis, increasing the yield of the extractive process with CO_2 and it obtains relevant compounds such as Artepillin C (HPPC) and p-coumaric acid [2,24,36,38,49]. Table 2 shows the results for the content of phenolic compounds, flavonoids and IC_{50} (DPPH) using 1 and 2% ethanol (co-solvent) in relation to the CO_2 mass (m/m), and

Table 2. Determination of the content of phenolic compounds, flavonoids, IC_{50} (DPPH), 3,5-diprenyl-4-hydroxicinnamic acid (Artepillin C) and acid 4-hydroxycinnamic (p-coumaric acid) of the green propolis extracts obtained by supercritical extraction with CO_2 and co-solvent in different concentrations (50°C, 250 bar and CO_2 flux of 6 g/min).

Parameters co-solvent (m/m)	Analyses				
	Total Phenolic (mgEAG/g)	IC $_{50}$ (μg/ml)	Flavonoids (mg EQ/g)	HPPC (μg/mL)	P-coumaric acid (μg/mL)
0% ethanol	62.21±1.12^a	201.98±2.23^a	20.27±0.71^a	—	—
1% ethanol	80.3±1.68^b	145.25±3.62^b	26.53±0.67^b	546.89±20.09^a	34.82±2.01^a
2% ethanol	66.72±1.49^c	193.75±4.27^c	23.02±0.47^c	308.47±13.24^b	14.32±1.24^b

Values representing the same letter, on the same column, do not show significant differences (p>0.05) by the Tukey Test at 95% confidence.

doi:10.1371/journal.pone.0134489.t002

without it. Also, it shows the concentration of Artepillin C and p-coumaric acid in the presence of a co-solvent.

The addition of a co-solvent in the supercritical carbon dioxide can significantly improve the extraction [89]. From the results shown on Table 2, it can be demonstrated that the presence of the co-solvent on the SFE process increases the extraction capacity of the phenolic compounds, flavonoids and antioxidant activity (IC_{50}) of the green propolis under the conditions used. The results for these parameters are significantly better on extracts using 1% ethanol, when compared to the process using 2%, or to its total absence. Furthermore, the concentration of Artepilllin C and p-coumaric acid was significantly superior with the presence of 1% ethanol in the system (m/m) (Table 2). Lee et al. [2] investigated the use of different concentrations of ethyl acetate (2 to 6% m/m) as a co-solvent to increase the efficiency of the extractive process of the Artepillin C in propolis samples. The best results were identified with the addition of 6% of co-solvent. The difference between the literature and the present work is, probably, due to differences in the extraction method and the characteristics of the raw material. Lin et al. [89] identified that the quantity of extractable oil from *Schisandra chinensis* seeds are significantly increased with the presence of ethanol as co-solvent. The assumption is that the role of the co-solvent in the extraction is, mainly, to decrease the thickness of the limit layer for mass transfer, considering that the increase in the quantity of ethanol in the system does not significantly increase the process yield.

A possible explanation for the improved extraction capacity with the presence of a lower quantity of ethanol (1% m/m) is that this substance has, in its molecules, the group OH, which make them capable of forming hydrogen bonds among themselves. On the other hand, the molecules of the polar components present in propolis also form hydrogen chains among themselves. Therefore, in order to the solute to be solvated by the solvent, the formation of new hydrogen bond is necessary, this time between the ethanol molecules and the molecules of the polar compounds of the propolis. The energy required to form these new connections is from the rupture between the hydrogen bonds and the solute molecules. However, when the quantity of co-solvent (% ethanol) is too high, there will not be enough energy to break the bonds between the ethanol molecules, and consequently less polar compounds will be solubilized by the solvent, causing a decrease in the extraction yield of these compounds and of the antioxidant activity [90].

Other studies evaluated the yield of the extraction process, depending on the percentage of co-solvent used in the system, and identified that, depending on the increase on the mass percentage of the co-solvent, there is no significant increase–and there may even be a decrease–on the quantity of the extract obtained. This, consequently, reduces the extraction of the relevant compounds in the matrix. Biscaia and Ferreira [25] compared the extraction yields of the propolis obtained through different procedures, including the extraction with supercritical CO_2 with (2.5 and 7%) and without the presence of ethanol (co-solvent). The maximum yield for the supercritical extraction process was obtained using intermediate conditions of ethanol (5% m/m). Marqués et al. [91] determined the best results for the extraction of antioxidant compounds of grape seeds by SFE (CO_2 supercritical), when used an approximate concentration of 3% (m/m) ethanol. Samples of Brazilian propolis and propolis from Taiwan were studied, using supercritical CO_2 and CO_2 with the addition of co-solvent (ethanol and water, 1:2 and 1:5 (m/v)). This was aimed at increasing the solubility in water and the anti-cancerous capacity of the extracts [49]. The addition of ethanol (1:5 m/v) in the process increased the extraction capacity of the nine phenolic compounds identified and quantified in the samples, whereas the addition of water did not show a positive influence in the process, when compared to extraction with only CO_2. Some patents also revealed that the propolis extracts obtained by SFE with CO_2 and ethanol improved the extraction efficiency of active compounds from this

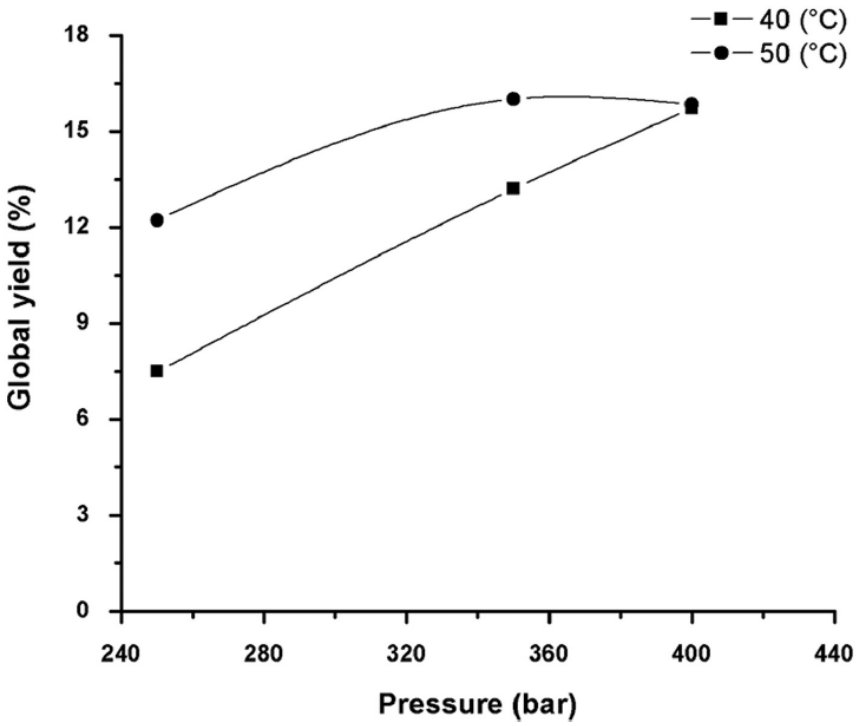

Fig 6. Global Yield Isotherm (%) for green propolis using CO_2 as supercritical fluid, ethanol as co-solvent (1% m/m) at temperature of 40 and 50°C and pressures of 250, 350 and 400 bar (CO_2 flux of 6.0 g/min; extraction time 2 h 30 min).

doi:10.1371/journal.pone.0134489.g006

matrix, including, for example, the Artepillin C and p-coumaric acid [92–93]. Similar results to this study were identified by Garmus et al. [94], Porto et al. [95], Bitencourt et al. [96] and Campos et al. [97], when evaluated different concentrations of the co-solvent in the extraction process.

Determination of global yield isotherms (GYIs)

In order to elaborate the GYIs, the best conditions of previous assays were used. That is, S/F value of 110, 1% co-solvent (ethanol m/m), CO_2 flow of 6.0g /min with pressure variations (250, 350 and 400 bar) and temperature (40 and 50°C). On Fig 6, the GYI for the extraction process of the green propolis are shown using the different conditions.

Under the pressures of 250 and 350 bar, the yield of the extraction process is significantly higher at temperature of 50°C when compared to 40°C. In these conditions the density of the solvent decreases considerably with the temperature, conducting to a lower solvation and also to a lower global yield. At 400 bar, the two isotherm curves crossover. At this point, there is no influence of the temperature on the global yield. This behaviour is, probably, an indication of the vicinity of the isotherm crossover, and was also identified by different studies reporting the competition between the two effects caused by the increase in temperature: (1) the reduction in the solvent power, with a decrease in yield, and (2) an increase in the pressure of the solute vapour, with an increase in yield [25,98–100]. In the study peformed with the Brazilian propolis at 200 bar, the yield was constant for all tested temperatures (30; 40; and 50°C) [25].

For the isotherm obtained at 40°C, it was observed that the global yield increases with the increase in pressure, whereas for the isotherm at 50°C, the global yield decreases with pressures above 350 bar. Therefore, there is no increase in power of extraction with the increase in vapour pressure at this point. On the isotherm at 40°C, the increase in pressure accelerates the mass transfer in the supercritical extractor, and increases the yield of extraction for the propolis components [36].

Therefore, the increase in density (which increases with pressure) increases the solvation power of the supercritical fluid, as well as the increase in temperature improves its diffusivity, the transfer of mass and consequently the extraction capacity [28]. However, high temperatures are not always advantageous, since they result in the reduction of the fluid's density, decreasing its solvent power [101]. For this reason, the definition of the adequate binomial pressure/temperature determines the success or otherwise of the extractive process. According to Meireles [102], the importance of measuring the global yield is due to the fact that this is an efficient methodology to select the best pressure and temperature conditions, according to the yield obtained in an initial phase of development in the supercritical extraction process. Therefore, for the raw material studied, the best global yield was obtained at pressure of 350 bar and temperature at 50°C (Fig 6).

Piantino et al. [103] identified the best global yield for leaves of *Baccharis dracunculifolia* at the higher temperature and pressure used (60°C and 400 bar), whereas the lowest global yield was obtained at lower conditions (40°C and 200 bar). Lin et al. [89] determined the pressure of 350 bar and temperature of 50°C as the best conditions applied for the extraction of *Schisandra chinensis* seeds oil. Besides, they observed the same behaviour identified for the green propolis, that is, when the temperature increases from 40 to 50°C, with constant pressure of 350 bar, there is a significant increase in the extraction yield.

On Fig 7, the isotherms for the yield of total phenolic compounds, flavonoids and antioxidant activity (IC_{50}) obtained under the different conditions analysed are showed. They present a similar profile to the global yield isotherm (Fig 6). The best results for the studied parameters were presented on the isotherm at 50°C, with a significant increase in the extraction of phenolic compounds, flavonoids and antioxidant activity (IC_{50}), when the pressure is increased from 250 to 350 bar. The increase in temperature at a fixed pressure reduces the density of the CO_2 in the supercritical state, reducing solubility. However, at the same time, it increases the vapour pressure of the compounds to be extracted. In this way, there is an increase in the tendency to turn these compounds into a fluid phase.

According to what was shown by the global yield isotherm at 50°C, the increase in pressure to 400 bar also represents a significant reduction in the quantification of the phenolic compounds and a reduction in the antioxidant activity. For the extraction of flavonoids, keeping the temperature constant at 50°C, the increase in pressure from 350 to 400 bar does not significantly influence the extraction yield of these compounds. This is probably related to the increase in solubility of the flavonoids in supercritical CO_2 at a higher pressure [104–106]. For the isotherm at 40°C, the increase in pressure increases the concentration of the compounds analysed and the antioxidant activity. This could be justified according to the fact that the solubility of the solute increases with the operation pressure at a constant temperature, due to the increase in the solvent density [37]. These results point to a temperature of 50°C and pressure of 350 bar as ideal conditions for the extraction of the propolis bioactive compounds.

It should be highlighted that the flavonoids are considered the most important compounds of propolis for determining the quality of this material. Zordi et al., [36], analysed multiple regression for obtainment of polyphenols in propolis extracts using supercritical CO_2. Considering the content of total flavonoids, they identified that the parameters of pressure and temperature had significant effects, where the higher yields for these compounds (>10%) were

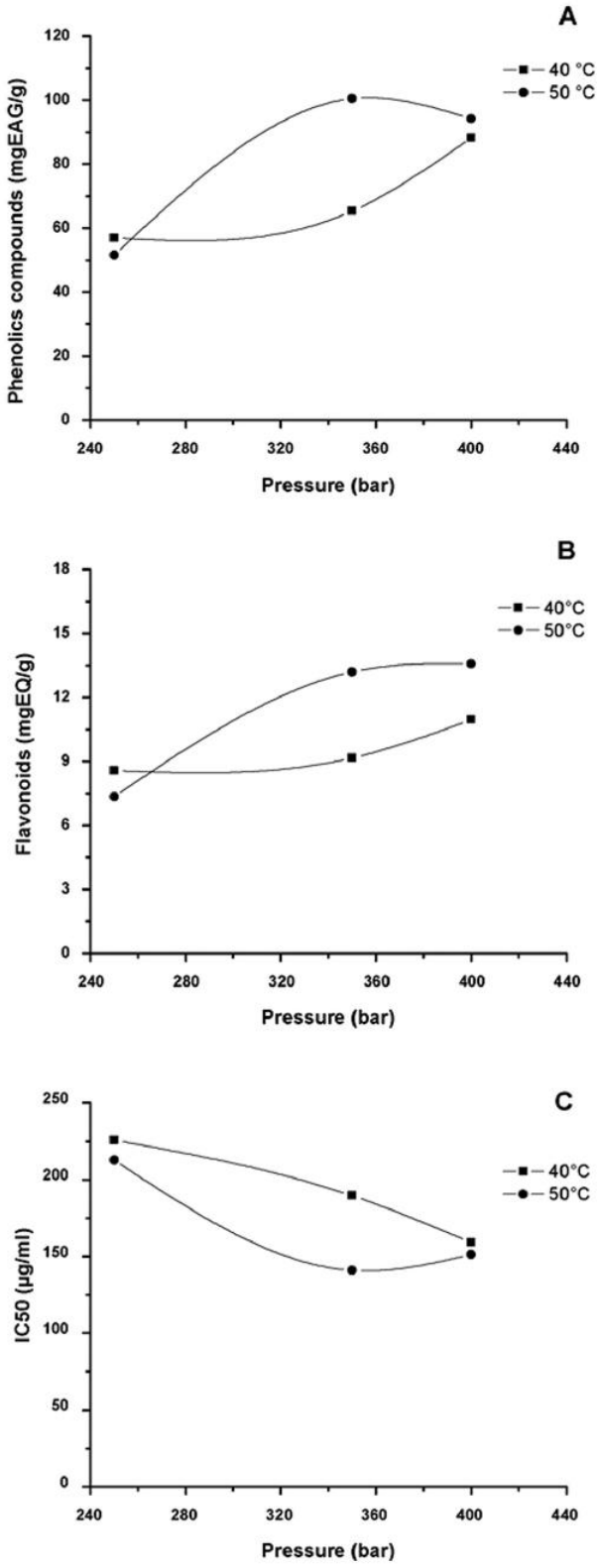
A
Phenolics compounds (mgEAG/g)
120
100
80
60
40
20
0
240
280
320
360
400
440
Pressure (bar)
40 °C
50 °C
B
Flavonoids (mgEQ/g)
18
15
12
9
6
3
0
240
280
320
360
400
440
Pressure (bar)
40°C
50°C
C
IC50 (µg/ml)
250
200
150
100
50
0
240
280
320
360
400
440
Pressure (bar)
40°C
50°C

Fig 7. Isotherms for (A) total phenolic compounds; (B) flavonoids; and (C) antioxidant activity (IC_{50}) for extraction of green propolis using CO_2 as supercritical fluid, ethanol as co-solvent (1% m/m) at temperatures of 40 and 50°C and pressures of 250, 350 and 400 bar (CO_2 flux of 6.0 g/min; extraction time 2 h 30min).

doi:10.1371/journal.pone.0134489.g007

observed at 270 and 200 bar at 40°C. You et al. [49] evaluated the extraction of flavonoids in propolis samples and identified that the extraction yield increased with pressure (276–345 bar), with the best yield point (10,5% w/w) being obtained under the conditions 345 bar and 45°C, very close to those conditions used in this study.

The influence of pressure and temperature for the extraction of p-coumaric acid and Artepillin C (μg/ml) are presented in Fig 8. On Table 3 are expressed the results obtained for the concentration of these two markers in μg/ml and g/100g for each extract, and also the results for the global yield (S6 Fig). These results identified these two markers of green propolis according to the isotherms presented on Figs 6 and 7.

For the p-coumaric acid (Fig 8A), the behaviour is the same presented by the global yield isotherm (Fig 6) at pressure 400 bar, where both isotherm curves crossover. There is, however, no influence of temperature on the extraction yield of this compound (to 400 bar pressure). In the two isotherms, an increase in concentration of the p-coumaric acid is observed when the pressure is increased from 250 to 350 bar. Besides that, the increase in temperature represents an increase in the yield of the compound at the constant pressures of 250 and 350 bar, justified through the increase in the vapour pressure, considering the simple effect. For Artepillin C, a similar behaviour to that of the p-coumaric acid is observed with the increase in temperature and pressure up to 350 bar. That means there is an increase in concentration of the compound. In higher pressures (400 bar), the concentration of the Artepillin C decreased with the increase temperature. In the other hand, in lower pressures (250 and 350 bar), the opposite behaviour was observed, it means that the concentration increases with the increase temperature and constant pressure. This type of behaviour can be explained based on the effect of temperature and pressure on the variation in density of the solvent, and due to the effect of temperature on the vapour pressure of the solute [81].

The isotherms crossover at 370 to 375 bar (Fig 8). In this region, the effect of temperature on the increase of vapour pressure compensated for the effect of temperature on the decrease in density of the solvent. Due to the existence of this point, the lower and higher concentrations occurred in the same isotherm of 50°C. For the bixin samples, Silva et al. [107] determined the crossover pressure at 280 bar, whereas Albuquerque and Meireles [48] identified it at 200 bar. A similar behaviour to that of this study for the extraction of Artepillin C and p-coumaric acid was identified by Piantino et al. [103], using the conditions of 300 bar and 40 and 50°C, and by Paviani et al. [81] using the conditions of 240 bar and 20, 35 and 50°C. That shows that the increase temperature promotes a higher yield of these compounds at a determined constant pressure.

The phenolic compound Artepillin C (HPPC), with confirmed important biological activities, was present in higher concentration in the extracts obtained at 50°C and 350 bar, showing 8.93±0.01 g of the compound in 100 g of propolis extract. Values inferior to those used in this study for Artepillin C content were found by Chen et al. [38] (1.27 g/100g) in samples of Brazilian propolis by SFE, under the conditions of 207 bar, 60°C and 6% ethyl acetate co-solvent; by Biscaia and Ferreira [25] (0.457 g/100g) under the conditions 150 bar, 40°C and 5% ethanol as co-solvent; and by Paviani et al. [81] (0.524 g/100g) under the conditions of 250 bar, 50°C and 5% of ethanol as co-solvent. The variations found are related to the origin of the matrix [108], as well as the conditions of process used.

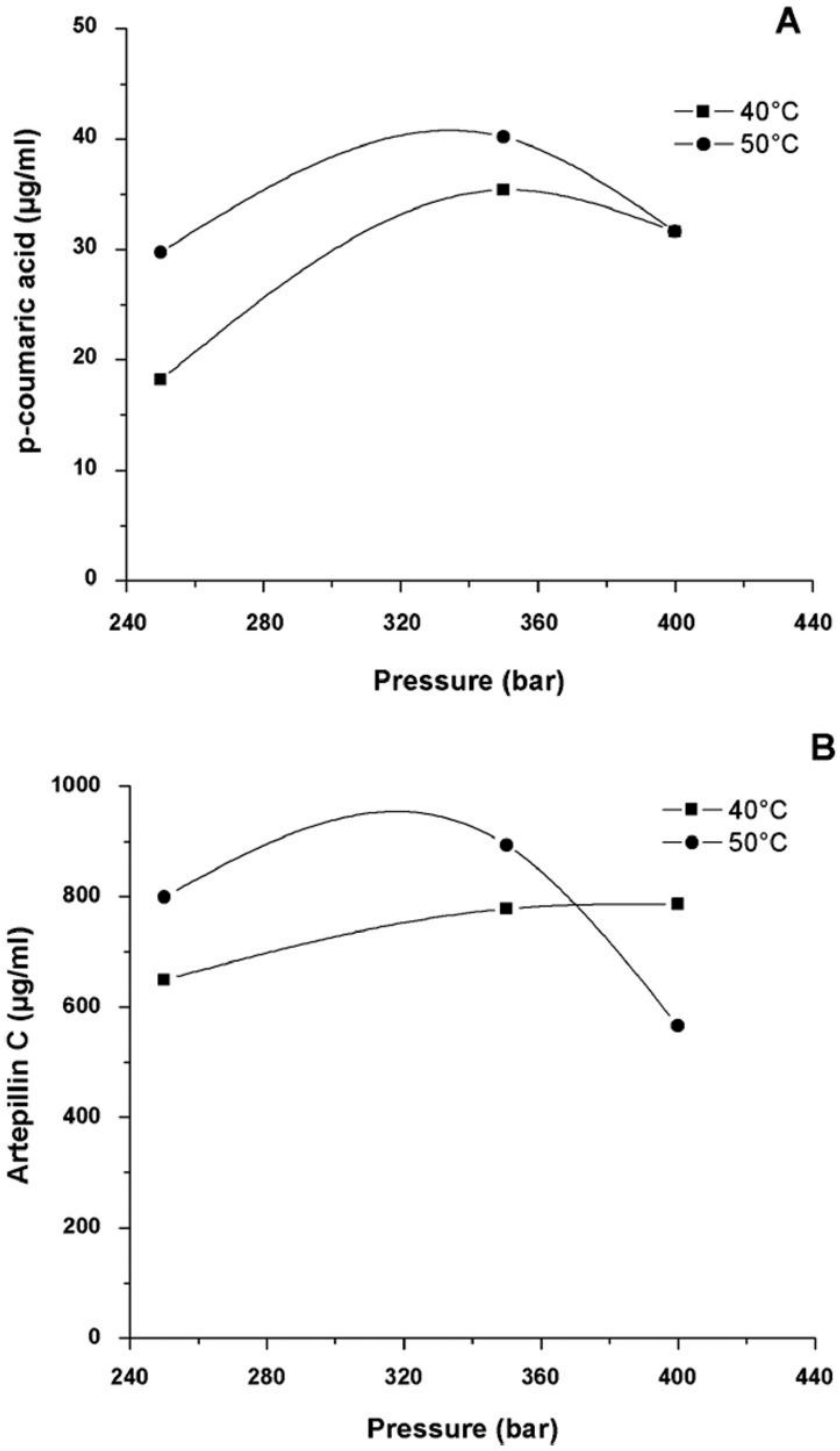

Fig 8. Isotherms for (A) concentration of p-coumaric acid; and (B) Artepillin C; on green propolis extracts obtained using CO_2 as supercritical fluid, ethanol as co-solvent (1% m/m) at temperatures of 40 and 50°C and pressures of 250, 350 and 400 bar (CO_2 flux of 6.0 g/min; time of extraction 2 h 30 min).

doi:10.1371/journal.pone.0134489.g008

Table 3. Determination of the acid 4-hydroxycinnamic (p-coumaric acid) content and 3,5-diprenyl-4-hydroxicinnamic acid (Artepillin C) and global yield values of the green propolis extracts obtained by supercritical extraction at different temperatures and pressures (mean ± standard deviation).

Conditions of Temperature (°C) and Pressure (bar)	P-cumaric Acid (μg/ml)	P-coumaric Acid (g/100g)	Artepillin C (μg/ml)	Artepillin C (g/100g)	Global yield (%)
T = 40 P = 250	18.20±0.63[a]	0.18±0.01[a]	649.10±6.69[a]	6.49±0.06[a]	7.48±0.12[a]
T = 50 P = 250	29.76±3.59[b]	0.29±0.03[b]	799.32±3.46[b]	7.99±0.03[b]	12.22±0.13[b]
T = 40 P = 350	35.39±0.55[c]	0.35±0.01[c]	778.04±1.32[c]	7.78±0.13[c]	13.02±0.07[c]
T = 50 P = 350	40.18±5.26[d]	0.40±0.05[d]	893.08±1.62[d]	8.93±0.01[d]	16.02±0.53[d]
T = 40 P = 400	31.60±2.46[b]	0.31±0.02[b]	786.23±8.16[b]	7.86±0.08[b]	15.73±0.14[e]
T = 50 P = 400	31.62±3.84[b]	0.31±0.03[b]	565.92±7.09[e]	5.66±0.07[e]	15.85±0.21[e]

Values showing the same letter, in a same column, do not present significant differences (p>0.05) according to Tukey Test at 95% confidence.

doi:10.1371/journal.pone.0134489.t003

Finally, it is demonstrated that the effect of pressure on the kinetics of extraction using supercritical CO_2 is already well established in the literature. There is no consensus within the scientific community to support that an increase in the operating pressure would have a positive effect in the extraction rate of biologically active compounds, considering the behaviour of each sample [109–110]. However, if the increase in pressure acts positively, increasing the extraction capacity to a constant temperature, the financial viability to work at a high pressure has to be analysed case by case, as any increase of pressure is directly related to an increase of energy consumption.

In the case of obtaining biologically active compounds from green propolis, working with high pressures does not seem economically convenient, considering that the extraction capacity of the relevant compounds is decreased. This is especially true for Artepillin C, the main

Fig 9. Raw propolis sample obtained after supercritical extraction using CO_2 and ethanol as co-solvent.

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marker for green propolis [38,103], and presently considered as a compound of great interest to the pharmaceutic industry.

On Fig 9, an image of propolis sample after SFE with CO_2 and ethanol as co-solvent is shown. It is consensus in the literature that extracts obtained by SFE present a different profile when compared to that obtained by conventional extractions, such as the alcoholic extraction or that by Soxhlet [2,10,38,111]. For propolis, despite a lower yield when compared to alcoholic extraction, the extracts obtained by SFE are considered as highly valuable products, considering the profile of compounds shown. According to Biscaia and Ferreira [25], the viability of the process is directly related to the quality of the final product, in order to improve the biological potential of the raw material. Lee et al. [2] extracted Artepillin C from the Brazilian green propolis samples using supercritical CO_2 modified with a co-solvent, followed by chromatography in column, in order to obtain very pure Artepillin C. Furthermore, the propolis residue, after extraction by SFE, can be used for the traditional extraction, producing a good quantity of flavones and phenols [36].

Conclusion

This study presented important aspects regarding the process parameters for obtaining green propolis extracts using supercritical CO_2. Considering that propolis is a complex material, the use of extraction with supercritical fluids, especially CO_2, is extremely attractive to obtain extracts with high added value, despite the low yield of the process. In this study, it was identified that the addition of co-solvent in the system is an advantage to improve the extraction process of the biologically active compounds present in the green propolis, due to the hydrophilic characteristic of the active compounds. The best extracts were obtained in the conditions of 50°C, 350 bar, SF 110 and the presence of 1% ethanol as co-solvent. In these conditions, the extracts have a high content of phenolic compounds, high antioxidant activity (represented by the low IC_{50} obtained), as well as a high flavonoid content. The concentration of Artepillin C obtained in these conditions is very important, which is demonstrated by the obtainment of extracts with important biological applications, already evidenced in other studies, as well being as a product of interest for food, pharmaceutic and cosmetic industries.

Supporting Information

S1 Fig. The equipment used to obtain the extracts of green propolis was a pilot unity called SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc.).
(DOCX)

S2 Fig. Mounting the extraction cell (100 ml capacity): The ethanol was homogenised with the sample and placed in the extraction cell, together with wool and glass pearls to fill the cell.
(DOCX)

S3 Fig. Green propolis extracts obtained by supercritical extraction.
(DOCX)

S4 Fig. Ultraviolet spectrum to the p-coumaric acid standard at 290 nm by HPLC.
(DOCX)

S5 Fig. Ultraviolet spectrum to the Artepillin C standard at 290 nm by HPLC.
(DOCX)

S6 Fig. Chromatogram Green propolis extract obtained by supercritical extraction in the conditions of 50°C, 350 bar.
(DOCX)

S1 Table. Extraction for green propolis in relation to the Mean DPPH (%) and Mean Total Phenolic (%) obtained at different times for obtaining the pilot kinetic (Conditions: 40°C, 100 bar, CO_2 flux of 6 g/min and 7.5 g of green propolis). (Table support of Fig 3).
(DOCX)

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Author Contributions

Conceived and designed the experiments: BASM SBN MAU-G FFP. Performed the experiments: BASM GAB ASC SSC RPDS DFS HNB JLCR. Analyzed the data: BASM HNB MAU-G FFP. Contributed reagents/materials/analysis tools: BASM SBN MAU-G FFP. Wrote the paper: BASM HNB MAU-G FFP SSC RPDS.

References

1. Ghisalberti EL. Propolis: A review. Bee World. 1979; 60 (2): 59–84. Available: http://www.ibra.org.uk/articles/Propolis-A-Review.
2. Lee YN, Chen CR, Yang HL, Lin CC, Chang CMJ. Isolation and purification of 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) in brazilian propolis by supercritical fluid extractions. Purif Technol. 2007; 54(1): 130–138. doi: 10.1016/j.seppur.2006.08.028
3. Costa AS, Machado BAS, Umsza-Guez MA, Cirqueira MG, Nunes SB, Padilha FF. Survey of studies with propolis produced in the state of Bahia, Brazil. Sitientibus: Biol Sci. 2013; 13: 1–7. doi: 10.13102/scb324
4. Hata T, Tazawa S, Ohta S, Rhyu MR, Misaka T, Ichihara K. Artepillin C, a major ingredient of brazilian propolis, induces a pungent taste by activating TRPA1 channels. PLoS One. 2012; 7(11): e48072. doi: 10.1371/journal.pone.0048072 PMID: 23133611
5. Frozza COS, Garcia CSC, Gambato G, Souza MDO, Salvador M, Moura S, et al. Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. Food Chem Toxicol. 2013; 52: 137–142. doi: 10.1016/j.fct.2012.11.013 PMID: 23174518
6. Campos JF, Santos UP, Macorini LFB, Melo AMMF, Balestieri JBP, Paredes-Gamero EJ, et al. Antimicrobial, antioxidant and cytotoxic activities of propolis from *Melipona orbignyi* (Hymenoptera, Apidae). Food Chem Toxicol. 2014; 65: 374–380. doi: 10.1016/j.fct.2014.01.008 PMID: 24412556.
7. Griffiths AG, Toshitaka U, Fumihiko T, Daisuke H. Effect of vapors from fractionated samples of propolis on microbial and oxidation damage of rice during storage. J Food Eng. 2008; 88: 341–352. doi: 10.1016/j.jfoodeng.2008.02.019
8. Silva JCS, Rodrigues S, Feás X, Estevinho LM. Antimicrobial activity, phenolic profile and role in the inflammation of propolis. Food Chem Toxicol. 2012; 50(5): 1790–1795. doi: 10.1016/j.fct.2012.02.097 PMID: 22425940
9. Nedji N, Loucif-Ayad W. Antimicrobial activity of Algerian propolis in foodborne pathogens and its quantitative chemical composition. Asian Pac J Trop Dis. 2014; 4(6): 433–437. doi: 10.1016/S2222-1808(14)60601-0
10. Wang K, Zhang J, Ping S, Ma Q, Chen X, Xuan H, et al. Anti-inflammatory effects of ethanol extracts of Chinese propolis and buds from poplar (*Populus×canadensis*). J Ethnopharmacol. 2014; 155(1): 300–311. doi: 10.1016/j.jep.2014.05.037 PMID: 24882729

11. Funakoshi-Tago M, Okamoto K, Izumi R, Tago K, Yanagisawa K, Narukawa Y, et al. Anti-inflammatory activity of flavonoids in Nepalese propolis is attributed to inhibition of the IL-33 signaling pathway. Int Immunopharmacol. 2015; 25(1): 188–198. In Press. doi: 10.1016/j.intimp.2015.01.012

12. Gressler LT, Silva AS, Machado G, Rosa LD, Dorneles F, Gressler LT, et al. Susceptibility of *Trypanosoma evansi* to propolis extract in vitro and in experimentally infected rats. Res Vet Sci. 2012; 93(3): 1314–1317. doi: 10.1016/j.rvsc.2012.02.007 PMID: 22405907

13. Rasouli M, Keshavarz M, Kalani M, Asaei S. The effect of propolis ethanol extract on humoral and cellular responses of mice immunized with *Leishmania* recombinant GP63 protein. Int J Infect Dis. 2012; 16(1) e312–e313. doi: 10.1016/j.ijid.2012.05.1010

14. Sforcin JM, Bankova V. Propolis: Is there a potential for the development of new drugs? J Ethnopharmacol. 2011; 133(2): 253–260. doi: 10.1016/j.jep.2010.10.032 PMID: 20970490

15. Chan GC, Cheung KW, Sze DM. The immunomodulatory and anticancer properties of propolis. Clin Rev Allergy Immunol. 2013; 44(3): 262–273. doi: 10.1007/s12016-012-8322-2 PMID: 22707327

16. Franchi GC Junior, Moraes CS, Toreti VC, Daugsch A, Nowill AE, Park YK. Comparison of effects of the ethanolic extracts of Brazilian propolis on human leukemic cells as assessed with the MTT assay. Evid Based Complement Alternat Med. 2012; 2012: 1–6. doi: 10.1155/2012/918956

17. Choudhari MK, Haghniaz R, Rajwade JM, Paknikar KM. Anticancer activity of Indian stingless bee propolis: an in vitro study. Evid Based Complement Alternat Med. 2013; 2013: 1–10. doi: 10.1155/2013/928280

18. Novak EM, Silvia MSC, Marcucci MC, Sawaya ACHF, López BGC, Fortes MAHZ, et al. Antitumoural activity of Brazilian red propolis fraction enriched with xanthochymol and formononetin: an in vitro and in vivo study. J Funct Foods. 2014; 11: 91–102. doi: 10.1016/j.jff.2014.09.008

19. Begnini KR, Leon PMM, Thurow H, Schultze E, Campos V, Rodrigues FM, et al. Brazilian Red Propolis Induces Apoptosis-Like Cell Death and Decreases Migration Potential in Bladder Cancer Cells. Evid Based Complement Alternat Med. 2014; 2014: 1–13. doi: 10.1155/2014/639856

20. Ito J, Chang FR, Wang HK, Park YK, Ikegaki M, Kilgore N, et al. Anti-HIV activity of moronic acid derivatives and the new melliferone-related triterpenoid isolated from Brazilian propolis. J Nat Prod. 2001; 64(10): 1278–1281. doi: 10.1021/np010211x PMID: 11678650.

21. Kai H, Obuchi M, Yoshida H, Watanabe W, Tsutsumi S, Park YK, et al. In vitro and in vivo anti-influenza virus activities of flavonoids and related compounds as components of Brazilian propolis (AF-08). J Funct Foods. 2014; 8: 214–223. doi: 10.1016/j.jff.2014.03.019

22. Massaro FC, Brooks PR, Wallace HM, Nsengiyumva V, Narokai L, Russell FD. Effect of Australian Propolis from Stingless Bees (*Tetragonula carbonaria*) on Pre-Contracted Human and Porcine Isolated Arteries. PLoS One. 2013; 8(11): e81297. doi: 10.1371/journal.pone.0081297 PMID: 24260567

23. Teles F, Silva TM, Cruz FP Júnior, Honorato VH, Oliveira Costa H, et al. Brazilian Red Propolis Attenuates Hypertension and Renal Damage in 5/6 Renal Ablation Model. PLoS One. 2015; 10(1): e0116535. doi: 10.1371/journal.pone.0116535 PMID: 25607548

24. Catchpole OJ, Grey JB, Mitchel KA, Lan JS. Supercritical antisolvent fractionation of propolis tincture. J Supercrit Fluids. 2004; 29(1–2): 97–106. doi: 10.1016/S0896-8446(03)00033-0

25. Biscaia D, Ferreira SRS. Propolis extracts obtained by low pressure methods and supercritical fluid extraction. J Supercrit Fluids. 2009; 51(1): 17–23. doi: 10.1016/j.supflu.2009.07.011

26. Silva-Carvalho R, Miranda-Gonçalves V, Ferreira AM, Cardoso SM, Sobral AJFN, Almeida-Aguiar C, et al. Antitumoural and antiangiogenic activity of Portuguese propolis in in vitro and in vivo models. J Funct Foods. 2014; 11: 160–171. doi: 10.1016/j.jff.2014.09.009

27. Matsushige K, Basnet P, Kadota S, Namba T. Potent free radical scavenging activity of dicaffeoyl quinic acid derivatives from propolis. J Tradi Med. 1996; 13: 217–228. Available: http://www.wakan-iyaku.gr.jp/wp-content/uploads/pdf/19960303_060622152233.pdf.

28. Erdogan S, Ates B, Durmaz G, Yilmaz I, Seckin T. Pressurized liquid extraction of phenolic compounds from Anatolia propolis and their radical scavenging capacities. Food Chem Toxicol. 2011; 49 (7): 1592–1597. doi: 10.1016/j.fct.2011.04.006 PMID: 21530603

29. Russo A, Longo R, Vanella A. Antioxidant activity of propolis: role of caffeic acid phenethyl ester and galangin. Phytotherapy. 2002; 73(1): 21–29. doi: 10.1016/S0367-326X(02)00187-9

30. Kimoto T, Aga M, Hino K, Koya-Miyata S, Yamamoto Y, Micallef MJ, et al. Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. Anticancer Res. 2001; 21 (1A): 221–228. PMID: 11299738.

31. Kimoto T, Arai S, Kohguchi M, Aga M, Nomura Y, Micallef MJ, et al. Apoptosis and suppression of tumor growth by artepillin C extracted from brazilian propolis. Cancer Detect Prev. 1998; 22(6): 506–515. doi: 10.1046/j.1525-1500.1998.00020.x PMID: 9824373.

32. Shimizu K, Swadesh KD, Hashimoto T, Sowa Y, Yoshida T, Sakai T, et al. Artepillin C in Brazilian propolis induces G0/G1 arrest via stimulation of Cip1/p21 expression in human colon cancer cells. Mol Carcinog. 2005; 44(4): 293–299. doi: 10.1002/mc.20148 PMID: 16224795.

33. Carvalho AA, Finger D, Machado CS, Schmidt EM, Costa PM, Alves APNN, et al. In vivo antitumoural activity and composition of an oil extract of brazilian propolis. Food Chem. 2011; 126(3): 1239–1245. doi: 10.1016/j.foodchem.2010.12.035

34. Guimarães NSS, Mello JC, Paiva JS, Bueno PCP, Berretta AA, Torquato RJ et al. *Baccharis dracunculifolia*, the main source of green propolis, exhibits potent antioxidant activity and prevents oxidative mitochondrial damage. Food Chem. Toxicol. 2012; 50(3–4):1091–1097. doi: 10.1016/j.fct.2011.11.014 PMID: 22119782

35. Marcucci MC, Rodriguez J, Ferrerez F, Bankova V, Groto R, Popov S. Chemical composition of Brazilian Propolis from São Paulo State. Z. Naturforsch., C, Biosci. 1998; 53c: 117–119. Available:http://zfn.mpdl.mpg.de/data/Reihe_C/53/ZNC-1998-53c-0117.pdf.

36. Zordi N, Cortesi A, Kikic I, Moneghini M, Solinas D, Innocenti G, et al. The supercritical carbon dioxide extraction of polyphenols from propolis: a central composite design approach. J Supercrit Fluids. 2014; 95:491–498. doi: 10.1016/j.supflu.2014.10.006

37. Reverchon E, Marco I. Supercritical fluid extration and fractionation of natural matter. J Supercrit Fluids. 2006; 38(2): 146–166. doi: 10.1016/j.supflu.2006.03.020

38. Chen CR, Lee YN, Lee MR, Chang CMJ. Supercritical fluids extraction of cinnamic acid derivatives from brazilian propolis and the effect on growth inhibition of colon cancer cells. J Taiwan Inst Chem Eng. 2009; 40(2): 130–135. doi: 10.1016/j.jtice.2008.07.014

39. Paviani LC, Dariva C, Marcucci MC, Cabral FA. Surpercritical cabon dioxide selectivity to fracionate phenolic compounds from the dry ethanolic extract of propolis. J Food Process Eng. 2010; 33(1): 15–27. doi: 10.1111/j.1745-4530.2008.00256.x

40. Capuzzo A, Maffei ME, Occhipinti A. Supercritical fluid extraction of plant flavors and fragrances. Molecules. 2013; 18(6): 7194–7238. doi: 10.3390/molecules18067194 PMID: 23783457

41. Gomes PB, Mata VG, Rodrigues AE. Production of rose geranium oil using supercritical fluid extraction. J Supercrit Fluids. 2007; 41(1): 50–60. doi: 10.1016/j.supflu.2006.08.018

42. Pourmortazavi SM, Hajimirsadeghi SS. Supercritical fluid extraction in plant essential and volatile oil analysis. J Chromatogr. 2007; 1163(1–2): 2–24. doi: 10.1016/j.chroma.2007.06.021

43. Machado BAS, Pereira CG, Nunes SB, Padilha FF, Umsza-Guez MA. Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives. Sep Sci Technol. 2013; 48(3): 2741–2760. doi: 10.1080/01496395.2013.811422

44. Mendiola JA, Herrero M, Cifuentes A, Ibá-es E. Use of compressed fluids for sample preparation: food applications. J Chromatogr. 2007; 8(1152): 234–46. doi: 10.1016/j.chroma.2007.02.046

45. Association of Official Analytical Chemists (AOAC). Official methods of analysis of AOAC International. 16th ed. Washington: AOAC International; 1997.

46. Blight EG, Dyer WJ. A Rapid Method of Total Lipid Extraction and Purification. Can J Biochem. 1959; 31: 911–917. PMID: 13671378.

47. Van Soest PJ, Wine RH. Use of detergents in analysis of fibrous feeds. In: Determination of plant cell wall constituents. J Assoc Off Anal Chem. 1967; 50: 50. Available: http://catalogo.latu.org.uy/doc_num.php?explnum_id=1418

48. Albuquerque CLC, Meireles MAA. Defatting of annatto seeds using supercritical carbon dioxide as a pretreatment for the production of bixin: experimental, modeling and economic evaluation of the process. J Supercrit Fluids. 2012; 66: 86–95. doi: 10.1016/j.supflu.2012.01.004

49. You GS, Lin SC, Chen CR, Tsai WC, Chang CJ, Huang WW. Supercritical carbon dioxide extraction enhances flavonoids in water-soluble propolis. J Chin Inst Chem Eng. 2002; 33(3): 233–241. Available: http://ejournal.stpi.narl.org.tw/NSC_INDEX/KSP/journalView/volumnView.jsp?journalId=J000004&volumnId=V000383&journalSetId=.

50. Meireles MAA. Extraction of bioactive compounds from Latin American plants. In: Martinez JL, editor. Supercritical Fluid Extraction of Nutraceuticals and Bioactive Compounds. CRC Press/Taylor & Francis Group. 2008. pp. 243–274.

51. Daugsch A. The red propolis of northeast Brazil and its chemical and biological characteristics. D.Sc. Thesis, State University of Campinas. 2007. Available: http://www.bibliotecadigital.unicamp.br/document/?code = vtls000406573

52. Brazil. Ministry of Health. National Health Surveillance Agency (ANVISA). Resolution n° 899, of may 29, 2003: Guide to the validation of analytical and bioanalytical methods. Available: http://portal.anvisa.gov.br/wps/wcm/connect/4983b0004745975da005f43fbc4c6735/RE_899_2003_Determina

+a+publica%C3%A7%C3%A3o+do+Guia+para+valida%C3%A7%C3%A3o+de+m%C3%A9todos+anal%C3%ADticos+e+bioanal%C3%ADticos.pdf?MOD=AJPERES.

53. National Institute of Metrology, Standardization and Industrial Quality (INMETRO). Guidelines for Chemical Testing Methods Validation. 2011. Available: http://www.inmetro.gov.br/Sidoq/Arquivos/Cgcre/DOQ/DOQ-Cgcre-8_04.pdf.

54. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965; 16(3): 144–158. Available: http://ajevonline.org/content/16/3/144.

55. Singleton VL, Orthofer R, Lamuela-Ravento´s RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of FolinCiocalteu reagent. Methods Enzymol. 1999; 299: 152–178. doi: 10.1016/S0076-6879(99)99017-1

56. Peschel W, Sánchez-Rabaneda F, Diekmann W, Plescher A, Gartzia I, Jiménez D, et al. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. Food Chem. 2006; 97 (1):137–150. doi: 10.1016/j.foodchem.2005.03.033

57. Marcucci MC, Ferreres F, Guarcía-Viguera C, Bankova VS, Castro SL, Dantas AP, et al. Phenolic compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol. 2001; 74(2): 105–112. doi: 10.1016/S0378-8741(00)00326-3 PMID: 11167028.

58. Brand-William W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebensm Wiss Technol. 1995; 28: 25–30. doi: 10.1016/S0023-6438(95)80008-5

59. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol. 2004; 26: 211–219. Available: http://rdo.psu.ac.th/sjstweb/journal/26-2/07-DPPH.pdf.

60. Moreira TF. Chemical composition of propolis: vitamins and aminoacids. Braz Rev Farmacogn. 1986; 1(1): 12–19. doi: 10.1590/S0102-695X1986000100003

61. Bonvehi JS, Coll FV, Jordá RE. The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem Soc. 1994; 71(5): 529–532. doi: 10.1007/BF02540666

62. Funari CS, Ferro VO. Propolis analysis. Braz J Food Technol. 2006; 26(1): 171–178. doi: 10.1590/S0101-20612006000100028

63. Mendonça LS. Environmental, chemical and biological aspects related to the red propolis. M.Sc Thesis, Tiradentes University. 2011. Available: http://psa.unit.br/wp-content/uploads/2012/04/2011_Lucyana_Mendon%C3%A7a.pdf.

64. Dias LG, Pereira AP, Estevinho LM. Comparative study of different Portuguese samples of propolis: Pollinic, sensorial, physicochemical, microbiological characterization and antibacterial activity. Food Chem Toxicol. 2012; 50(12): 4246–4253. doi: 10.1016/j.fct.2012.08.056 PMID: 22981908

65. Attia YA, Abd Al-Hamid AE, Ibrahim MS, Al-Harthi MA, Bovera F, Elnaggar ASh. Productive performance, biochemical and hematological traits of broiler chickens supplemented with propolis, bee pollen, and mannan oligosaccharides continuously or intermittently. Livest Sci. 2014; 164: 87–95. doi: 10.1016/j.livsci.2014.03.005

66. Bankova V. Chemical diversity of Propolis and the problem of standardization. J Ethnopharmacol. 2005; 100: 114–117. doi: 10.1016/j.jep.2005.05.004 PMID: 15993016.

67. Falcão SI, Tomás A, Vale N, Gomes P, Freire C, Vilas-Boas M. Phenolic quantification and botanical origin of Portuguese propolis. Ind Crops Prod. 2013; 49: 805–812. doi: 10.1016/j.indcrop.2013.07.021

68. Wilson MB, Brinkman D, Spivak M, Gardner G, Cohen JD. Regional variation in composition and antimicrobial activity of US propolis against *Paenibacillus* larvae and *Ascosphaera apis*. J Invertebr Pathol. 2015; 124: 44–50. doi: 10.1016/j.jip.2014.10.005 PMID: 25450740

69. Brazil. Ministry of Health. National Health Surveillance Agency (ANVISA). Normative Instruction n° 3, of January 19, 2001: Technical Regulations of identify and Quality of bee venom, royal Bee, Jelly Wax, Lyophilized Royal Jelly, Bee Pollen, Propolis and Propolis Extract. Available: http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao = visualizar&id=1798.

70. Lewicki PP. Water as the determinant of food engineering properties. A review. J Food Eng. 2004; 61 (4): 483–495. doi: 10.1016/S0260-8774(03)00219-X

71. Aguiar SC, Cottica SM, Boeing JS, Samensari RB, Santos GT, Visentainer JV, et al. Effect of feeding phenolic compounds from propolis extracts to dairy cows on milk production, milk fatty acid composition, and the antioxidant capacity of milk. Anim Feed Sci Technol. 2014; 193: 148–154. doi: 10.1016/j.anifeedsci.2014.04.006

72. Kandiel MMM, El-Asely AM, Radwan HA, Abbass AA. Modulation of genotoxicity and endocrine disruptive effects of malathion by dietary honeybee pollen and propolis in Nile tilapia (Oreochromis niloticus). J Adv Res. 2014; 5(6): 671–684. doi: 10.1016/j.jare.2013.10.004 PMID: 25685536

73. Tylkowski B, Trusheva B, Bankova V, Giamberini M, Peev G, Nikolova A. Extraction of biologically active compounds from propolis and concentration of extract by nanofiltration. J Memb Sci. 2010; 348(1–2): 124–130. doi: 10.1016/j.memsci.2009.10.049

74. Budel JM, Duarte MR. Pharmacobotanical study of aerial vegetative parts of *Baccharis anomala DC.*, Asteraceae. Braz Rev Farmacogn. 2008; 18: 761–768. doi: 10.1590/S0102-695X2008000500022

75. Budel JM, Duarte MR, Santos CAM, Farago PV. Leaf and stem morpho-anatomy of *Baccharis dracunculifolia DC.*, Asteraceae. Acta Farm Bonaerense. 2004; 23(4): 477–483. Available: http://www.latamjpharm.org/trabajos/23/4/LAJOP_23_4_1_8_I54P2Y1561.pdf.

76. Bastos EMAF, Santana RA, Calaça-Costa AGF, Thiago PS. Interaction between *Apis mellifera* L. and *Baccharis dracunculifolia DC*, that favours green propolis production in Minas Gerais. Braz J Biol. 2011; 71(3): 727–734. doi: 10.1590/S1519-69842011000400018 PMID: 21881797

77. Ziémons E, Goffin E, Lejeune R, Cunha AP, Angenot L, Thunus L. Supercritical carbon dioxide extraction of tagitinin C from *Tithonia diversifolia*. J Supercrit Fluids. 2005; 33(1): 53–59. doi: 10.1016/j.supflu.2004.04.001

78. Mezzomo N, Martínez J, Ferreira SRS. Supercritical fluid extraction of peach (*Prunus persica*) almond oil: kinetics, mathematical modeling and scale-up. J Supercrit Fluids. 2009; 51(1): 10–16. doi: 10.1016/j.supflu.2009.07.008

79. Pardo-Castaño C, Velásquez M, Bola-os G. Simple models for supercritical extraction of natural matter. J Supercrit Fluids. 2015; 97: 165–173. doi: 10.1016/j.supflu.2014.09.044

80. Wang BJ, Lien YH, Yu ZR. Supercritical fluid extractive fractionation-study of the antioxidant activities of propolis. Food Chem. 2004; 86(4): 237–243. doi: 10.1016/j.foodchem.2003.09.031

81. Paviani LC, Saito E, Dariva C, Marcucci MC, Sánchez-Camargo AP, Cabral FA. Supercritical CO_2 extraction of raw propolis and its dry ethanolic extract. Braz J Chem Eng. 2012; 29(2): 243–251. doi: 10.1590/S0104-66322012000200005

82. Quispe-Condori S, Sánchez D, Foglio MA, Rosa PTV, Zetzl C, Brunner G, et al. Global yield isotherms and kinetic of artemisinin extraction from *Artemisia annua L* leaves using supercritical carbon dioxide. J Supercrit Fluids. 2005; 36(1): 40–48. doi: 10.1016/j.supflu.2005.03.003

83. Pereira CG, Rosa PVT, Meireles MAA, Extraction and isolation of indole alkaloids from *Tabernaemontana catharinensis A. DC*: technical and economical analysis. J Supercrit Fluids. 2007; 40(2): 232–238. doi: 10.1016/j.supflu.2006.07.001

84. Veggi PC, Cavalcanti RN, Meireles MAA. Production of phenolic-rich extracts from Brazilian plants using supercritical and subcritical fluid extraction: experimental data and economic evaluation. J Food Eng. 2014; 131: 96–109. doi: 10.1016/j.jfoodeng.2014.01.027

85. Ferreira SRS, Meireles MAA. Modeling the supercritical fluid extraction of black pepper (*Piper nigrum L.*) essential oil. J Food Eng. 2002; 54(4): 263–269. doi: 10.1016/S0260-8774(01)00212-6

86. Ferreira SRS, Nikolov ZL, Doraiswamy LK, Meireles MAA, Petenate AJ. Supercritical fluid extraction of black pepper (*Piper nigrun* L.) essential oil. J Supercrit Fluids. 1999; 14(3): 235–245. doi: 10.1016/S0896-8446(98)00092-8

87. Rodrigues VM, Sousa EMBD, Monteiro AR, Chiavone-Filho O, Marques MO, Meireles MAA. Determination of the solubility of extracts from vegetable raw materials in pressurized CO_2: a pseudo ternary mixture formed by cellulosic structure + solute + solvent. J Supercrit Fluids. 2002; 22(1): 21. doi: 10.1016/S0896-8446(01)00108-5

88. Robards K. Strategies for the determination of bioactive phenols in plants, fruit and vegetables. J Chromatogr A. 2003; 1000(1–2): 657–691. doi: 10.1016/S0021-9673(03)00058-X PMID: 12877194

89. Lin CH, Lin HW, Wu JY, Houng JY, Wan HP, Yang TY, et al. Extraction of lignans from the seed of *Schisandra chinensis* by supercritical fluid extraction and subsequent separation by supercritical fluid simulated moving bed. J Supercrit Fluids. 2015; 98: 17–24. doi: 10.1016/j.supflu.2014.12.025 In Press.

90. Morrison R, Boyd R. Organic Chemistry. 13nd ed. Lisboa: Calouste Gulbenkian Foundation; 1996.

91. Marqués JL, Porta GD, Reverchon E, Renuncio JAR, Mainar AM. Supercritical antisolvent extraction of antioxidants from grape seedsafter vinification. J Supercrit Fluids. 2013; 82: 238–243. doi: 10.1016/j.supflu.2013.07.005

92. Wu J, Luyu Q. Supercritical multiple extraction of bee glue. 2000. Chinese Patent, CN1258511.

93. Machado BAS, Padilha FF, Nunes SB, Guedes CMC, Costa AS, Umsza-Guez MA, et al. Process for obtaining 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) propolis extract using supercritical carbon dioxide and co-solvent. 2014. Patente brasileira BR1020140320121.

94. Garmus TT, Paviani LC, Queiroga CL, Cabral FA. Extraction of phenolic compounds from pepper-rosmarin (*Lippia sidoides Cham.*) leaves by sequential extraction in fixed bed extractor using supercritical

CO2, ethanol and water as solvents. J Supercrit Fluids. 2015; 99: 68–75. In Press. doi: 10.1016/j.supflu.2015.01.016

95. Porto C, Natolino A, Decorti D. Extraction of proanthocyanidins from grape marc by supercritical fluid extraction using CO_2 as solvent and ethanol–water mixture as co-solvent. J Supercrit Fluids. 2014; 87: 59–64. doi: 10.1016/j.supflu.2013.12.013

96. Bitencourt RG, Queiroga CL, Montanari Í Junior, Cabral FA. Fractionated extraction of saponins from Brazilian ginseng by sequential process using supercritical CO2, ethanol and water. J Supercrit Fluids. 2014; 92: 272–281. doi: 10.1016/j.supflu.2014.06.009

97. Campos LMAS, Leimann FV, Pedrosa RC, Ferreira SRS. Free radical scavenging of grape pomace extracts from cabernet sauvingnon (*Vitis vinifera*). Bioresour Technol. 2008; 99(17): 8413–8420. doi: 10.1016/j.biortech.2008.02.058 PMID: 18445523

98. Michielin EMZ, Bresciani LFV, Danielski L, Yunes RA, Ferreira SRS. Composition profile of horsetail (*Equisetum giganteum L.*) oleoresin: comparing SFE and organic solvents extraction. J Supercrit Fluids. 2005; 33(2): 131–138. doi: 10.1016/j.supflu.2004.07.004

99. Danielski L, Michielin EMZ, Ferreira SRS. Horsetail (Equisetum giganteum L.) oleoresin and supercritical CO_2: experimental solubility and empirical data correlation. J Food Eng. 2007; 78(3): 1054–1059. doi: 10.1016/j.jfoodeng.2005.12.016

100. Kitzberger C, Lomonaco R, Michielin E, Danielski L, Correia J, Ferreira SRS. Supercritical fluid extraction of shiitake oil: curve modeling and extract composition. J Food Eng. 2009; 90(1): 35–43. doi: 10.1016/j.jfoodeng.2008.05.034

101. Fattori M, Bulley NR, Meisen A. Carbon dioxide extraction of canola seed: oil solubility and effect of seed treatment. J Am Oil Chem Soc. 1988; 65(6): 968–974. doi: 10.1007/BF02544522

102. Meireles MAA. Supercritical extraction from solid: process designer data (2001–2003). Curr Opin Solid State Mater Sci. 2003; 7: 321–330. doi: 10.1016/j.cossms.2003.10.008

103. Piantino CR, Aquino FWB, Follegatti-Romero LA, Cabral FA. Supercritical CO2 extraction of phenolic compounds from *Baccharis dracunculifolia*. J Supercrit Fluid. 2008; 47(2):209–214. doi: 10.1016/j.supflu.2008.07.012

104. Li Q, Zhang Z, Zhong C, Liu Y Liu, Zhou Q. Solubility of solid solutes in supercritical carbon dioxide with and without cosolvents. Fluid Phase Equilibria. 2003; 207(1–2): 183–192. doi: 10.1016/S0378-3812(03)00022-0

105. Machmudah S, Kawahito Y, Sasaki M, Goto M. Supercritical CO_2 extraction of roseship seed oil: fatty acids composition and process optimization. J Supercrit Fluids. 2007; 41(3): 421–428. doi: 10.1016/j.supflu.2006.12.011

106. Machmudah S, Kondo M, Sasaki M, Goto M, Munemasa J, Yamagata M. Pressure effect in supercritical CO_2 extraction of plant seeds. J Supercrit Fluids. 2008; 44(3): 301–307. doi: 10.1016/j.supflu.2007.09.024

107. Silva GF, Gamarra FMC, Oliveira AL, Cabral FA. Extraction of bixin from annatto seeds using supercritical carbon dioxide. Braz J Chem Eng. 2008; 25(2): 419–426. doi: 10.1590/S0104-66322008000200019 ISSN 0104-6632.

108. Park YK, Paredes-Guzman JF, Aguiar CL, Alencar SM, Fujiwara FY. Chemical constituents in *Baccharis dracunculifolia* as the main botanical origin of southeastern Brazilian propolis. J Agric Food Chem. 2004; 52(5): 1100–1103. doi: 10.1021/jf021060m PMID: 14995105.

109. Fiori L. Supercritical extraction of grape seed oil at industrial-scale: Plant and process design, modeling, economic feasibility. Chem Eng Process, 2010; 49(8): 866–872. doi: 10.1016/j.cep.2010.06.001

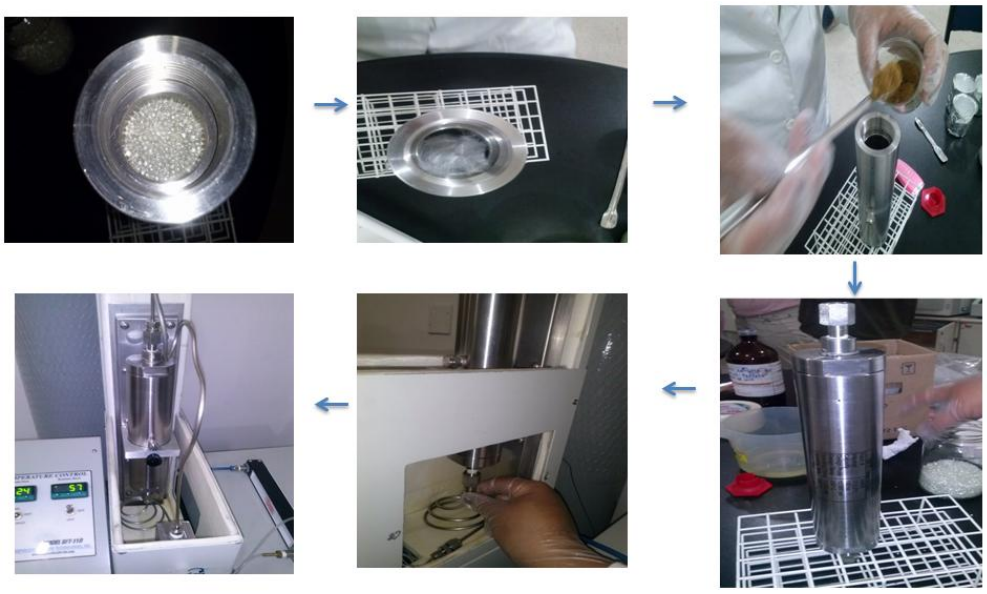
110. Duba KS, Fiori L. Supercritical CO_2 extraction of grape seed oil: effect of process parameters on the extraction kinetics. J Supercrit Fluids. 2015; 98: 33–43. doi: 10.1016/j.supflu.2014.12.021 In Press.

111. Chao-Rui C, Ying-Nong L, Chieh-Ming JC, Miau-Rong L, I-Chien W. Hot-pressurized fluid extraction of flavonoids and phenolic acids from Brazilian propolis and their cytotoxic assay in vitro. J Chinese Inst Chem Eng. 2007; 38(3–4): 191–196. doi: 10.1016/j.jcice.2007.04.004

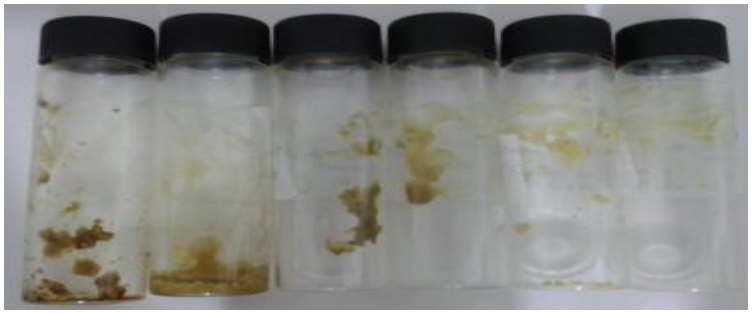
Supporting Information

http://journals.plos.org/plosone/article?id=10.1371/journal.pone.0134489

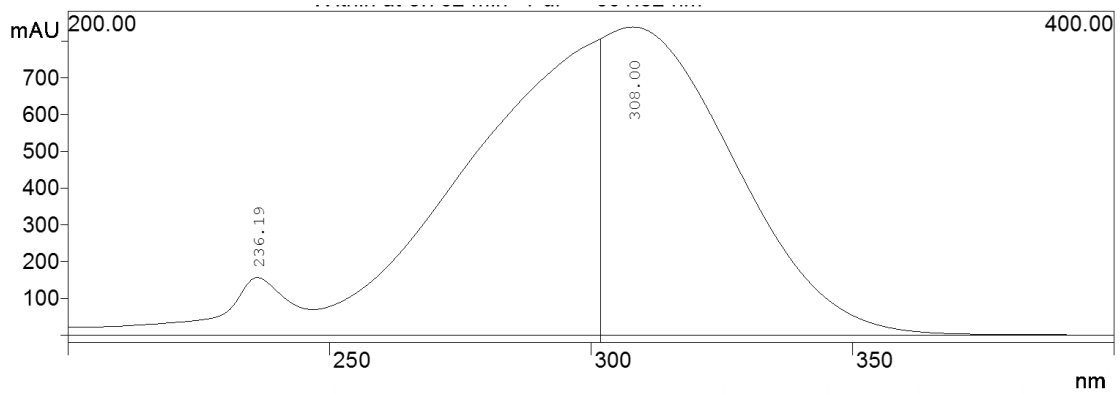
S1 Fig. The equipment used to obtain the extracts of green propolis was a pilot unity called SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc.). doi:10.1371/journal.pone.0134489.s001



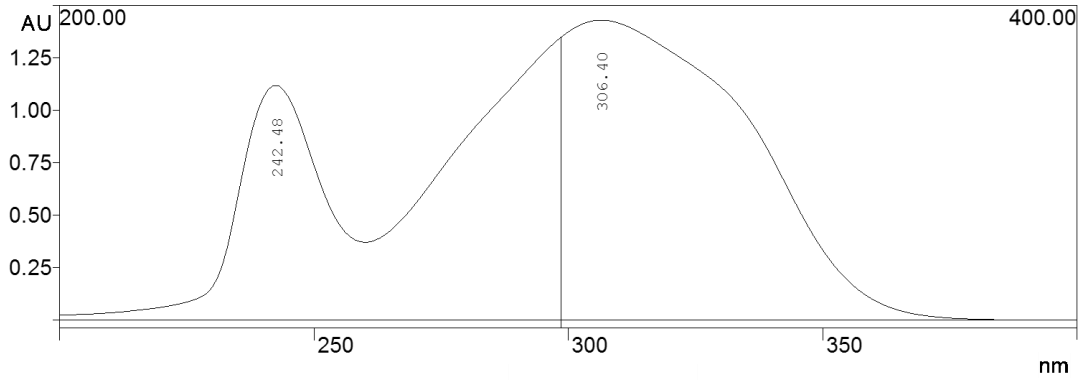
S2 Fig. Mounting the extraction cell (100 ml capacity): The ethanol was homogenised with the sample and placed in the extraction cell, together with wool and glass pearls to fill the cell. doi:10.1371/journal.pone.0134489.s002



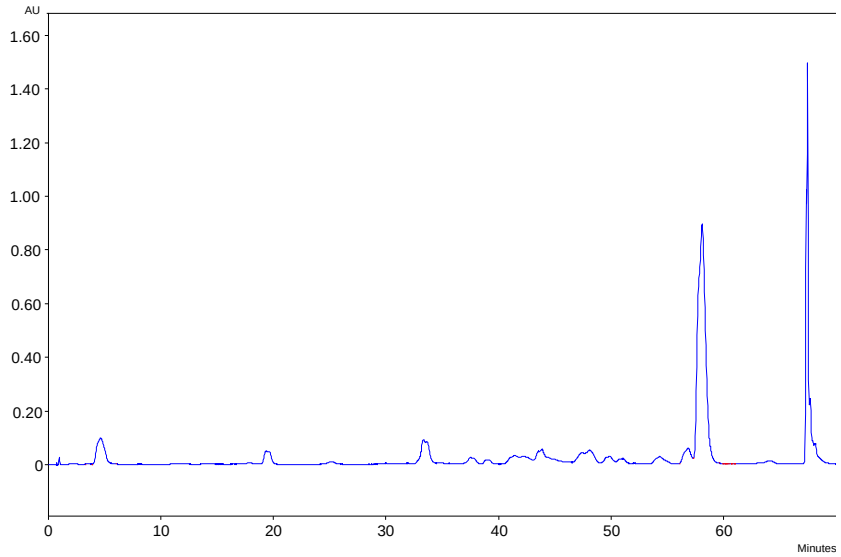
S3 Fig. Green propolis extracts obtained by supercritical extraction. doi:10.1371/journal.pone.0134489.s003



S4 Fig. Ultraviolet spectrum to the p-coumaric acid standard at 290 nm by HPLC. doi:10.1371/journal.pone.0134489.s004



S5 Fig. Ultraviolet spectrum to the Artepillin C standard at 290 nm by HPLC. doi:10.1371/journal.pone.0134489.s005



S6 Fig. Chromatogram Green propolis extract obtained by supercritical extraction in the conditions of 50°C, 350 bar. doi:10.1371/journal.pone.0134489.s006

S1 Table. Extraction for green propolis in relation to the Mean DPPH (%) and Mean Total Phenolic (%) obtained at different times for obtaining the pilot kinetic (Conditions: 40°C, 100 bar, CO_2 flux of 6 g/min and 7.5 g of green propolis). (Table support of Figure 3). doi:10.1371/journal.pone.0134489.s007

Time	Mean DPPH (%)	Mean Total Phenolic (%)
8,68	19,31086958	13,69099195
18,395	26,0097048	22,23532621
29,43	27,60353439	19,98892847
45,685	31,29300396	24,84275216
58,68	32,46429991	28,13212028
78,5	34,11263008	27,20949264
100,5	38,61335717	33,90857161
121,5	39,21922055	39,56468021
150,5	37,95633896	30,85988896
187,5	36,45508113	29,73669009
224,5	39,67360928	36,55611181
252,5	40,65314066	41,7709637
288,5	47,17560177	39,6449087

Capítulo 6. Artigo

7.0 CAPÍTULO 6: Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil

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RESEARCH ARTICLE

Chemical Composition and Biological Activity of Extracts Obtained by Supercritical Extraction and Ethanolic Extraction of Brown, Green and Red Propolis Derived from Different Geographic Regions in Brazil

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Abstract

The variations in the chemical composition, and consequently, on the biological activity of the propolis, are associated with its type and geographic origin. Considering this fact, this study evaluated propolis extracts obtained by supercritical extraction (SCO_2) and ethanolic extraction (EtOH), in eight samples of different types of propolis (red, green and brown), collected from different regions in Brazil. The content of phenolic compounds, flavonoids, *in vitro* antioxidant activity (DPPH and ABTS), Artepillin C, p-coumaric acid and antimicrobial activity against two bacteria were determined for all extracts. For the EtOH extracts, the anti-proliferative activity regarding the cell lines of B16F10, were also evaluated. Amongst the samples evaluated, the red propolis from the Brazilian Northeast (states of Sergipe and Alagoas) showed the higher biological potential, as well as the larger content of antioxidant compounds. The best results were shown for the extracts obtained through the conventional extraction method (EtOH). However, the highest concentrations of Artepillin C and p-coumaric acid were identified in the extracts from SCO_2, indicating a higher selectivity for the extraction of these compounds. It was verified that the composition and biological activity of the Brazilian propolis vary significantly, depending on the type of sample and geographical area of collection.

(Bahia). The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

Introduction

Propolis is characterized as a complex and resinous mixture produced by bees (*Apis melífera*) through the collection of variable vegetable sources [1–4]. Propolis is constituted by a variety of chemical compounds, including the derivatives of cinnamic acid, such as p-coumaric acid and Artepillin C, substituted benzoic acids, phenolic acids, flavonoids and aminoacids [5–7]. Different studies have already proved that the chemical composition of the propolis, and consequently its biological effects, depends on various factors such as the geographic origin, types of vegetable sources, time of collection and season of the year [8–10]. Although propolis is considered a complex mixture, its biological activities are reported due to the presence of the flavonoids, phenolic acids and ethers in the propolis composition [11–12].

Currently, the Brazilian propolis can be classified in 13 different types, according to its physical-chemical properties and the geographic area where it was found. Park et al. [6] classified the propolis samples collected from different regions around Brazil in 12 groups, according to appearance and colour of the extracts. The *Baccharis dracunculifolia* DC (Asteraceae) [13–14], a native plant from Brazil, is the most important botanical source of propolis in the Brazilian southeast, known as green propolis. Afterwards, a new propolis was found in hives located alongside the coast and mangroves in the Brazilian northeast and it was classified as a propolis of the group 13. This propolis is called red propolis, with botanical origin from *Dalbergia ecastophyllum* (L.) Taub. (Fabaceae) [15–16].

Propolis extracts are more commonly obtained through conventional techniques, such as the ethanolic extraction, aqueous extraction or by Soxhlet [17–19]. In the last few years, different studies showed the extraction with supercritical fluid (SFE) as an important alternative method to obtain compounds derived from natural matrices [20–23], including, for example, propolis [24–26]. This process shows advantages over the conventional ones, such as higher selectivity, reduction in use of organic solvents, obtaining extracts with high biological value and use of carbon dioxide (CO_2) as extractor solvent [27–29]. According to Machado et al. [30], when it is compared the extracts obtained by SFE with other conventional extractive methods, it is noted that the quantity of compounds obtained by SFE from the same matrix is very superior. However, despite the higher number of compounds extracted, often the yield of the extraction process is lower, which could indicate a higher selectivity.

Distinct biological properties and chemical compositions are described for the samples of propolis collected in Brazil, which is explained by the great Brazilian biodiversity. The antimicrobial and antitumoral capacity of red propolis were evidenced by many authors [18,31–32]. Different studies identified antifungal [33], immunomodulation [34–35], anti-ulcer [19,36] and anti-inflammatory [37] properties for samples of Brazilian green propolis. Fernandes et al. [38] evaluated the antioxidant effects and the (anti)genotoxicity in samples of brown propolis from the Brazilian savanna. In the study performed by Wilson et al. [39], the antimicrobial activity and chemical composition of 12 samples of propolis collected in different regions of the United States were evaluated. The profiles obtained by chromatography, as well as the activity of the microorganisms tested showed very distinct aspects, indicating that the variation of results was due to the geographical region. In view of that, the objective of this study was to perform the chemical characterization, evaluate the antioxidant capacity and antimicrobial activity of propolis extracts obtained by SFE and ethanolic extraction (conventional), as well as *in vitro* evaluation of antitumor of the ethanolic extracts against the cell lines of B16F10, from eight samples (brown, green and red) collected in different geographical regions of Brazil.

Material and Methods

Materials and reagents

Ethanol (HPLC degree) and aseptic acid (HPLC degree) were obtained from Merck Co. (Darmstadt, Germany) and methanol (PA) from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). A cellulose ester membrane filter of 0.45 μm (SLCR025NS, Millipore® Co., Bedford, Massachusetts, USA) was used. The carbon dioxide (CO_2) used in the extraction had 99.9% purity (White Martins Gases Industrials–São Paulo, Brazil). The standard 3,5-diprenil-4-hidroxicinamic (Artepillin C–cas number 72944-19-5) was acquired from Wako Pure Chemical Industries, Ltd. (Osaka, Japan) and the Acid 4-hidroxicinamic (p-coumaric acid–cas number 501-98-4), 2,2-diphenyl-1-picrylhydrazyl (DPPH), Acid Gallic (cas number 149-91-7), Quercetin (cas number 117-39-5), 2,2′-azinobis-(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) (cas number 30931-67-0) and (±)-6-Hydroxy-2,5,7,8-tetramethylchromane-2-carboxylic acid (Trolox) (cas number 53188-07-1) were acquired from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA).

Obtaining and processing of propolis samples

Approximately 700–1000g of propolis samples were donated by the company Apis Nativa Produtos Naturais (Prodapys–Santa Catarina–Brazil), originated from the different regions in Brazil, during the period of July to September 2013. Two samples of red propolis were from the Brazilian northeast (Alagoas and Sergipe), three samples of brown propolis from the south (Santa Catarina, Rio Grande do Sul and Parana) and three samples of green propolis were from the south and southeast of Brazil (Parana and Minas Gerais) (two samples from different regions) (Table 1). The samples of propolis were crushed in a grinder (Cadence–Brazil) and then sieved (60 mesh), in order to obtain an adequate granulometry (approximately 0.250 mm) to increase the surface area and homogenise the start material in the extraction processes. Small quantities (250g) of propolis were kept in a fridge at -10°C, in bottles protected with laminated paper in inert atmospheric conditions (N_2) in order to avoid degradation of the material.

Characterization of raw material

The determinations of humidity, protein and total ash contents were made according to the official methods of Association of Official Agricultural Chemists (AOAC) [40]. The total lipids were extracted and quantified through the cold extraction method described by Bligh & Dyer [41]. The determination of the mineral content was made in a digital flame photometer (DM-62, DIGIMED, São Paulo—Brazil) and the fiber content was obtained through the automatic

Table 1. Identification of propolis samples from different regions of Brazil and analysed in this study.

Sample Identification	State and region of Brazil	Colour Type
SER	Sergipe–Northeast	Red
RAL	Alagoas–Northeast	Red
GMG_1	Minas Gerais–Southeast	Green
GMG_2	Minas Gerais–Southeast	Green
GPR	Paraná –South	Green
BSC	Santa Catarina–South	Brown
BRS	Rio Grande do Sul–South	Brown
BPR	Paraná –South	Brown

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fibre analyser (A-220, ANKON, New York–USA) [42]. The quantification of the water activity took place using a decagon LabMaster (Novasina, Lachen–Switzerland), with electrolytic cell CM-2. The analyses were performed in triplicate. The Scanning electron microscopy (SEM) was performed in a scanning electron microscope JEOL JSM-6390LV (USA). After drying in an oven (105°C/45 min), the sample of crushed propolis was fixed manually using a tweezer (PELCO® Tweezers) of aluminum metal surfaces covered with carbon double-sided tape, called stubs. Because of the need for interaction of the electron beam with the sample, it was performed by coating deposition of metallic gold ions (sputtering). The sample was metalized in gold in a "Sputter oater" from Balzers, model SCD 50 (20nm). Then the stubs containing the metallic samples were stored in plastic boxes (storage boxes), duly sealed with parafilm (PARAFILM® M) to prevent moisture absorption. After 24 hours of rest, the samples were analyzed at different magnifications (Voltage 12 kV, Working Distance 12 mm, Spot size 44, Vacuum Mode HV).

Obtaining propolis extracts by supercritical fluid extraction (SFE) and low pressure extraction (LPE)

Supercritical extracts using CO_2 as supercritical fluid. The equipment used for obtaining the propolis extracts was the pilot unity called SFT-110 Supercritical Fluid Extractor (Supercritical Fluid Technologies, Inc.), composed by a high pressure bomb (capacity of up to 10,000 psi), extraction cell (capacity of 100 ml), furnace (containing a pre-warmer), static/dynamics valve and restrictor valve, flow meter, flux totalizer (ITRÓN, ACD G1.0, Argentina) and CO_2 cylinder. A CO_2 cylinder with fishing tube was used to ensure that only CO_2 in liquid state was used in the system, a requirement of the SFT-110. The CO_2 was not re-used in the system.

The extraction cell consisted of a packaging using 7.5 g of homogenised propolis sample with 1% ethanol co-solvent (m/m), wool and glass pearls, aimed at avoiding the preferred paths of CO_2 and the total filling of the bed. The extraction conditions were: pressure of 350 bar, temperature 50°C, S/F of 110 (mass of $CO_{2[solvent]}$ / mass of $propolis_{[solute]}$), 1% co-solvent (ethanol m/m), flow of CO_2 of 6 g/min and total time of extraction 2 h 30 min [26,43]. The temperature of the restrictor valve was adjusted at 80°C for all extraction processes. The extracts were collected in glass vials of 50 ml, immersed in ice at room pressure. The vials containing the extracts were protected with aluminium foil in inert atmospheric conditions (N_2) in order to avoid degradation of the material, and kept at 5°C until analysis (Fig 1A).

Low pressure extraction (conventional extraction). The ethanolic extracts of propolis were prepared with the addition of 15 ml ethanol (80%) to 2g propolis. The extraction occurred at 70°C temperature for 30 minutes under constant agitation in a Shaker (MA 420/MARCONI–Brazil) incubator, at 710 rpm rotation. Following that, the extract was centrifuged (Centrifuge SIGMA 2–16 KL) at 8800 rpm for 11 min at 5°C. At the end of the centrifugation, the supernatant was transferred to a 50 ml beaker, and 10 ml ethanol (80%) was added to the tube residue, where centrifugation was repeated. The supernatants were homogenised and kept at 50°C until completely dry (Fig 1B). Afterwards, the extracts were stored in tubes covered in aluminium foil in inert atmospheric conditions (N_2) in order to avoid degradation of the material. The material was kept at 5°C until analysis [6].

Chromatographic analysis: identification and quantification of the 3,5-diprenil-4-hidroxicinamic (Artepillin C) and acid 4-hidroxicinamic (p-coumaric acid)

To the identification and quantification of the 3,5-diprenil-4-hidroxicinamic (Artepillin C) and acid 4-hidroxicinamic (p-coumaric acid) in the propolis extracts, firstly, solutions of

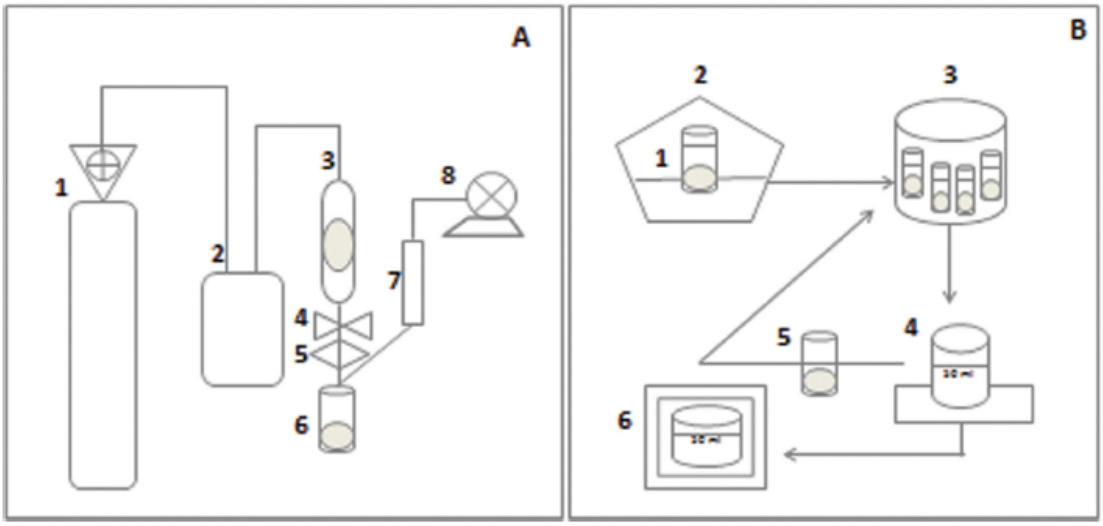

Fig 1. Extraction Process Stages. (A) High pressure extraction (SFE) 1 –CO_2 cylinder with fishing tube; 2 –Adjusted bomb at 350bar; 3 –Extraction cell using 7.5 g of sample (packed) and co-solvent at 50°C (furnace temperature); 4 –Static/dynamics valve; 5 –Restrictor valve adjusted to 6g/min CO_2 flow; 6 –Extracts into a collection bottle; 7 –Flow meter; 8 –Gas meter; (B) Low pressure extraction (conventional extraction) 1 –Propolis sample in ethanol (80%); 2 –Process extraction in a shaker (70°C, 30 minutes, 710rpm); 3 –Centrifugation at 8800rpm for 11 minutes at 5°C; 4 –Supernatant, centrifugation was repeated with the residue (10 ml of 80% ethanol); 6 –Homogenised supernatants and kept at 50°C until completely dry.

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10 mg/ml of propolis extracts obtained in the different conditions of the process were prepared and dissolved in ethanol and placed in ultrasound bath (Sanders, SONICLEAN 6 –Minas Gerais, Brazil—ANVISA 80273140001) for 30 minutes (Electronic timer microprocessor–Temperature 35°C electronically controlled and Ultrasound frequency 40 kHz). The exposure to the ultrasound system only started after reaching 35°C. The samples were filtered in a cellulose ester membrane filter 0.45 μm (Micropore®) for posterior injection in the High Performance Liquid Chromatograph (HPLC).

The chromatographic experiments were performed with the system HPLC EZChrom Elite, consisting of a VRW HITACHI L-2130 pump, equipped with an automatic injector and diode arrangement detector (DAD) VRW HITACHI L-2455 and oven VRW HITACHI L-2300. The chromatographic separation was based in the method proposed by Daugsch [15], adapted. The column LiChroCART Purospher StaR® RP18-e (75 mm x 4 mm i.d.) (3 μm) (Merck, Darmastad, Germany) was used together with a pre-column LiChroCART 4–4 LiChrospher 100RP18 (5 μm) from Merck.

The conditions for analysis were performed with an elution gradient with a mobile phase of aseptic acid 5% (aqueous phase) and methanol (organic phase) in different proportions and with total analysis time of 70 minutes (0 min-80:20; 10 min 70:30; 15 min-60:40; 30 min-50:50; 45 min-40:60; 60 min-30:70; 65 min-0:100; 70 min-80:20). The volume of injection was of 10 μL. The equipment was operated at room temperature (25±2°C). The reading of the diode arrangement detector was in the range of 200 to 400 nm and the chromatographic acquisition was defined at 290 nm. The identification of the compounds was performed through the comparison of time of retention and ultraviolet spectrum between the samples and the controls (standard). Aiming to ensure the reliability of the results obtained, a validation took place according to the National Health Surveillance Agency (ANVISA) [44] and National Institute of Metrology, Standardization and Industrial Quality (INMETRO) [45] methodologies. This

was done in accordance to the parameters of selectivity, linearity, precision, accuracy, detection limits and quantification limits.

Determination of the total phenolic compounds

The total phenolic content was determined using the Folin Ciocalteau reagent [46–47]. The reaction was prepared with a 0.5 ml aliquot of propolis extract (dissolved in ethanol aimed at obtaining a concentration of 200 μg/ml), 2.5 ml aqueous solution of Folin-Ciocalteau 10% and 2.0 ml sodium carbonate at 7.5%. The mixture was introduced in a thermo-regulated bath at 50°C for 5 minutes; afterwards, the absorbance was measured in a spectrophotometer LAMBDA 25 UV/vis Systems (PerkinElmer, Washington—USA) at 765 nm. The quantity of total phenolic was expressed as Gallic acid equivalents (EAG) (mg EAG/g of sample) through a calibration curve using known solutions to Gallic acid standard in the same conditions (λ = 765 nm). The Folin Ciocalteu method is associated to the appearance of a blue colouring due to the oxidation of phenols in basic medium [48].

Determination of flavonoid content

The determination of flavonoid content was performed through the reading in a spectrophotometer (LAMBDA 25 UV/Vis Systems—PerkinElmer USA) at 415 nm, using aluminium chloride at 2% in methanol [49] in a 1:1 solution (extract:aluminium chloride). The same procedure was performed using known solutions of quercetin standard to elaborate a standard curve. The quantity of total flavonoids was expressed as quercetin equivalents (EQ) (mg EQ/g of sample).

Determination of anti-oxidant activity *in vitro* (2,2-Diphenyl-1-picrylhydrazyl–DPPH)

The anti-oxidant activity *in vitro* of propolis extracts obtained in different conditions was evaluated using the reactive 2,2-Diphenyl-1-picrylhydrazyl (also known as the capacity to sequestrate the radical DPPH) [50–51]. Five dilutions of the extracts were prepared (20 to 400 μg/ml) in triplicates. An aliquot of 1 ml of each extract dilution was transferred to assay tubes with 3.0 ml of the ethanoic solution (Ethanol–absolute alcohol 99.8%) of the radical DPPH (0.004%). After 30 minutes incubation in the dark and at room temperature, the reduction of the free radical DPPH was measured through the reading of absorbance in 517 nm spectrophotometer (LAMBDA 25 UV/Vis Systems–PerkinElmer, Washington—USA).

The same procedure was performed with ethanol replacing the sample, considered blank. The capacity to sequestrate free radicals was expressed as the percentage of oxidation inhibition in the radical and calculated according to Eq 1. The IC_{50} value (necessary concentration of the extract to sequestrate 50% of DPPH radical) was calculated through the line equation based on the concentrations of extracts and its respective percentages of radical DPPH sequestration.

$$\%\ \text{sequestration} = 100 - [(\text{final absorbance of sample} * 100) / \text{blank absorbance}] \quad (1)$$

Determination of antioxidant activity *in vitro*: ABTS method (2,2′-azino-bis-3-ethylbenzthiazoline-6-sulphonic acid)

The ABTS assay was based on the van der Berg et al. [52] method, slightly modified by Kim et al. [53], with adaptations. Initially, a solution at 7 mM was prepared in distilled water. From this solution, an aliquot of 5 ml was removed and 88 μL of potassium persulfate at 2.45 mM

was added. The final product was incubated for 16 hours, protected from light in order to enable production of the radical cation $ABTS^{\bullet+}$. Afterwards, the solution was diluted in ethanol until it reached 700±50 absorbance, reading at 734 nm using a spectrophotometer. Originated from the formation of radicals, 20 μL aliquots of stock solutions of 1 $mg.ml^{-1}$, 0.75 $mg.ml^{-1}$, 0.5 $mg.ml^{-1}$ and 0.1 $mg.ml^{-1}$ taken from the extracts were added to 2 ml of the final solution of $ABTS^{\bullet+}$, and after 6 minutes of incubation the samples were read at 734 nm absorbance. The results were expressed in TEAC (antioxidant activity equivalent to Trolox (6-hydroxil-2,5,7,8-tetramethylchromo-2-carboxilic acid)) (Vitamin E).

Antimicrobial activity of the extracts EtOH and SCO_2

The antimicrobial activity of the extracts EtOH and SCO_2 was determined through the Minimal Inhibitory Concentration (MIC) and Minimal Bactericide Concentration (MBC) against *Staphylococcus aureus* (ATCC 33951 and 25923) and *Escherichia coli* (ATCC 25922), according to the *Clinical and Laboratory Standards Institute* [54] and Koo et al. [55]. The strains used were provided by the Bacteria Cultures Collection of the Instituto Oswaldo Cruz—FIOCRUZ (Manguinhos—Rio de Janeiro—Brazil) and initially reactivated in liquid BHI (Brain Heart Infusion) (Sigma-Aldrich Chemical Co.—St. Louis, MO, USA) at 37°C for 24h and then grown in BHI agar plates to inoculum preparation. After bacterial growth, the biomass was removed with the aid of bacteriological loops and suspended in 0.89% NaCl sterile solution, homogenizing the bacterial suspensions until turbidity equivalent to 0.5 McFarland standard scale (equivalent to concentration of 1.5x108 CFU/ml). The volume of 30 μl of the bacterial suspension was inoculated on 30 ml of BHI to give a bacterial concentration of 1-2x105 CFU/ml. In order to determine the MIC, the initial inoculum was 1-2x105 CFU/ml, and the concentrations of the extracts varied from 1600–3.1 μg.ml-1. The MIC was defined as the lowest concentration inhibiting bacterial growth (without visible growth) [55]. As a determining factor of MBC, the surface of the agar BHI (Brain Heart Infusion) was seeded with the samples which did not indicate the presence of visible bacterial growth. MBC was defined as the lowest concentration which did not allow any visible bacterial growth in agar [55]. The assays were realized in triplicate for each concentration of the extracts tested.

In vitro activity of the ethanolic extracts (EtOH) on the strains of tumoral cells B16F10

The cellular strain of murine melanoma, B16F10 (ATCC® CRL-6475™) was kept in a culture medium RPMI 1640 (Gibco®, Life Technologies, Carlsbad, CA, USA), complete with 10% foetal bovine serum (FBS) (Gibco®) and 1% antibiotic solution of penicillin/streptomycin. It was placed in an incubator at 37°C in environment an of 5% CO_2. The *in vitro* tests of the ethanolic extracts (EtOH) performed on these cells followed the procedure: a solution of 1% Trypsin-EDTA was used to detach the culture of confluent cells from the growth bottle; after 5 minutes the solution of trypsin was inactivated by FBS and after adding the culture medium the material was centrifuged for 10 minutes at 1500 RPM; after discarding the supernatant, the pellet of cells was again suspended in a complete RPMI medium and then the inoculum of cells corresponding to the final concentration of $1x10^5$ cells/ml was calculated. After distributing the culture medium in plaques of 24 wells, the compounds were added to two concentrations of 50 μg/ml and 100 μg/ml, and the DMSO was used as a diluting control and statistic parameter [56]. The cellular proliferation was determined after 24 and 48 hours of incubation using the method of colorimetric assay adapted from Busatti and Gomes [57], and reading by an ELISA (enzyme-linked immunosorbent assay) reader at 570 nm. The cells used in this study were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA).

Statistical analysis

The results were expressed in the form of mean ± standard deviation (n = 3). For the statistical analysis of the results, the programme Statistica® 6.0 from StatSoft (Tulsa, USA) was used. Variance analysis (ANOVA) and the Tukey test were used to identify significant differences among the means ($p>0.05$).

Results and Discussion

Characterization of raw propolis samples

Propolis is a complex mixture, containing resins, balsamic products, wax, essential oils, pollen, and microelements, besides other components. The samples presented a characteristic aroma, balsamic and/or resinous, malleable (red) to rigid (brown and green) consistencies at room temperature, with a very heterogeneous granulometry. It is noted that the analysis of the physical-chemical composition is of great importance to determine the quality of the studied material, considering the incorporation of this matrix in food products [58–60]. On Table 2, the results of the physical-chemical characterization of the different samples of propolis are shown. On Table 3 are the results for the content of certain minerals present in the samples.

The value of humidity and total solids varied from 6.90±0.05 (BRS) to 9.16±0.06% (BPR) and from 90.84±0.06 (BPR) to 93.10±0.05% (BRS), respectively, among the samples. Two samples of green propolis (GMG_1 and GMG_2) and one from brown propolis (BPR) were out of the required standards for the humidity content (maximum of 8%) [61]. As expected, the higher values of aw were identified on the samples with high humidity. In relation to the contents of ash, protein, lipids and fibres, a significant variation was observed among the samples ($p>0.05$), with results varying from 0.85±0.03 (BRS) to 3.30±0.11% (GMG_1), 0.84±0.01 (BRS) to 10.58±0.08 (GMG_1), 45.76±1.77% (GMG_1) to 74.31±5.69% (BSC) and 3.44±0.84 (RSE) to 51.39±1.03% (BRS), respectively (Table 2).

The determination of the total ash content is particularly important in samples of propolis commercialized in powder form, as this analysis can identify a possible adulteration of the material through the presence of impurities, or even residues from previously extracted propolis [6]. The samples were within the limit established by the Brazilian legislation (maximum 5%) [61]. Among the microelements analysed and identified in the samples, we can highlight the high contents of potassium on the three samples of green propolis (Table 3). Some studies show aluminium, vanadium, iron, calcium, silicon, manganese, strontium and potassium, as the main microelements present in propolis samples [5,49,62–64].

Table 2. Determination of the content of humidity, total solids, total ash, raw protein, total lipids, raw fibre and water activity (aw) of red, green and brown propolis samples collected in different regions of Brazil.

Sample	Humidity (%)	Total solids (%)	Total ash (%)	Protein (%)	Lipids (%)	Aw	Fiber (%)
RSE	7.26±0.99^{a}	92.74±0.99^{a}	1.04±0.11^{a}	1.72±0.01^{a}	65.74±2.63^{a}	0.690±0.01^{a}	3.44±0.84^{a}
RAL	7.03±0.42^{a}	92.97±0.42^{a}	0.96±0.03^{a}	2.30±0.05^{b}	66.33±0.01^{a}	0.689±0.01^{a}	7.66±0.90^{b}
GMG1	8.84±0.05^{b}	91.16±0.05^{b}	3.30±0.11^{b}	10.58±0.08^{c}	45.76±1.77^{b}	0.705±0.01^{b}	16.36±1.34^{c,f}
GMG2	9.03±0.48^{b,d}	90.97±0.48^{b,d}	3.24±0.17^{b}	9.83±0.97^{c}	47.33±4.82^{b,d}	0.704±0.01^{b}	15.92±1.03^{c}
GPR	7.13±0.12^{a}	92.87±0.12^{a}	3.15±0.03^{b}	9.98±0.83^{c}	48.72±1.29^{b,d}	0.688±0.02^{a}	20.89±1.39^{d}
BSC	7.07±0.10^{a}	92.93±0.10^{a}	1.73±0.19^{c}	3.90±0.49^{d}	74.31±5.69^{c}	0.657±0.02^{c}	7.29±0.30^{b}
BRS	6.90±0.05^{c}	93.10±0.05^{c}	0.85±0.03^{d}	0.84±0.01^{e}	74.08±4.08^{c}	0.674±0.01^{d}	51.39±0.14^{e}
BPR	9.16±0.06^{d}	90.84±0.06^{d}	2.38±0.20^{e}	6.90±0.02^{f}	49.53±1.70^{d}	0.755±0.01^{e}	18.11±1.07^{f}

Values showing the same letter on the same column do not show significant difference ($p>0.05$) through the Tukey test at 95% confidence level.

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Table 3. Quantification of minerals, sodium (Na), potassium (K), lithium (Li) and calcium (Ca) (mg/Kg), from the ash of propolis samples from different regions of Brazil.

Sample	Na (mg/Kg)	K (mg/Kg)	Li (mg/Kg)	Ca (mg/Kg)
SER	14.90±0.89^{a}	23.70±1.59$^{a.e}$	6.10±0.69^{a}	45.10±0.01^{a}
RAL	10.10±0.41^{b}	28.70±3.16^{a}	4.50±0.64^{b}	40.10±0.72^{b}
GMG1	2.40±0.01^{c}	399.1±4.91^{b}	1.80±0.01^{c}	9.00±0.01^{c}
GMG2	2.40±0.01^{c}	317.30±13.43^{c}	1.80±0.01^{c}	8.40±0.01^{d}
GPR	3.00±0.01^{d}	331.70±15.81^{c}	1.80±0.01^{c}	9.60±0.01^{e}
BSC	15.30±1.05^{a}	110.30±6.77^{d}	3.10±1.00^{d}	5.90±0.04^{f}
BRS	6.70±0.70^{e}	23.20±0.98^{e}	1.90±0.08^{e}	7.40±0.08^{g}
BPR	1.10±0.11^{f}	5.70±0.68^{f}	3.40±0.01^{d}	29.60±0.62^{h}

Values showing the same letter on the same column do not show significant difference ($p>0.05$) through the Tukey test at 95% confidence level.

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Performing a comparative evaluation of samples of the same type (in relation to colour), the lower variation identified for the physical-chemical parameters studied was for the red propolis (RSE and RAL). There are a few works reporting the total physical-chemical characterization of propolis, however the values obtained in this study for certain parameters are similar to those found in the literature [24,64–68]. The variation identified among the samples studied and with those from other studies is easily explained by the type of propolis, flora of the region and period of collection.

On Fig 2 the micrographics obtained for the different propolis samples are shown. No study evaluating the Brazilian propolis by SEM was identified. On the visual and microscopic analysis no strange substances were identified. In all images, it is possible to observe rugged surfaces covered by layers of wax and extractives. Similar characteristics were identified by Tylkowski et al., [69] for samples of propolis from Bulgaria. It is also important to note that there were similarities identified on the microscopic appearance (profile) of the samples of the same type (colour). For example, in all samples of green propolis, vegetable constituents were found, probably tector and/or glandular trichome and resinous substances from the vegetative apices of *Baccharis dracunculifolia* (Fig 2C, 2D and 2E) [7,70–72]. Elements which are similar to vegetable parts were also identified on the samples of brown propolis, which probably come from the flora visited by bees, such as species of *Copaifera* (Fig 2F, 2G and 2H) [73].

Determination of content for phenolic compounds, flavonoids and antioxidant activity of EtOH and SCO_2 activity

Table 4 shows the results for the content of total phenolic compounds, flavonoids and antioxidant activity of the extracts from different samples of propolis obtained by the two extraction methods (conventional ethanolic–EtOH and supercritical–SCO_2). The content of phenolic compounds varied from 97.97±0.01 (BPR SCO_2) to 300.36±0.01 mg EAG/g (RSE EtOH), whereas the content of flavonoids varied from 11.55±0.01 (BPR SCO_2) to 58.19±0.01 mg EQ/g (RAL EtOH) among other samples (Fig 3). There is great controversy in relation to the content of flavonoids present in samples of Brazilian propolis, in which phenolic acids are generally a lot more abundant. The antioxidant activity varied from 373.53±0.15 (BPR SCO_2) to 31.80 ±0.16 (GMG_1 EtOH) for DPPH (IC_{50}) and from 49.60±4.10 (BPR SCO_2) to 98.50±1.40 (RSE EtOH) for ABTS (Trolox 1 $mg.ml^{-1}$) (Fig 4).

It was verified that the extracts obtained from red propolis (RSE and RAL) originated from the Brazilian northeast showed the highest content of phenolic compounds and flavonoids.

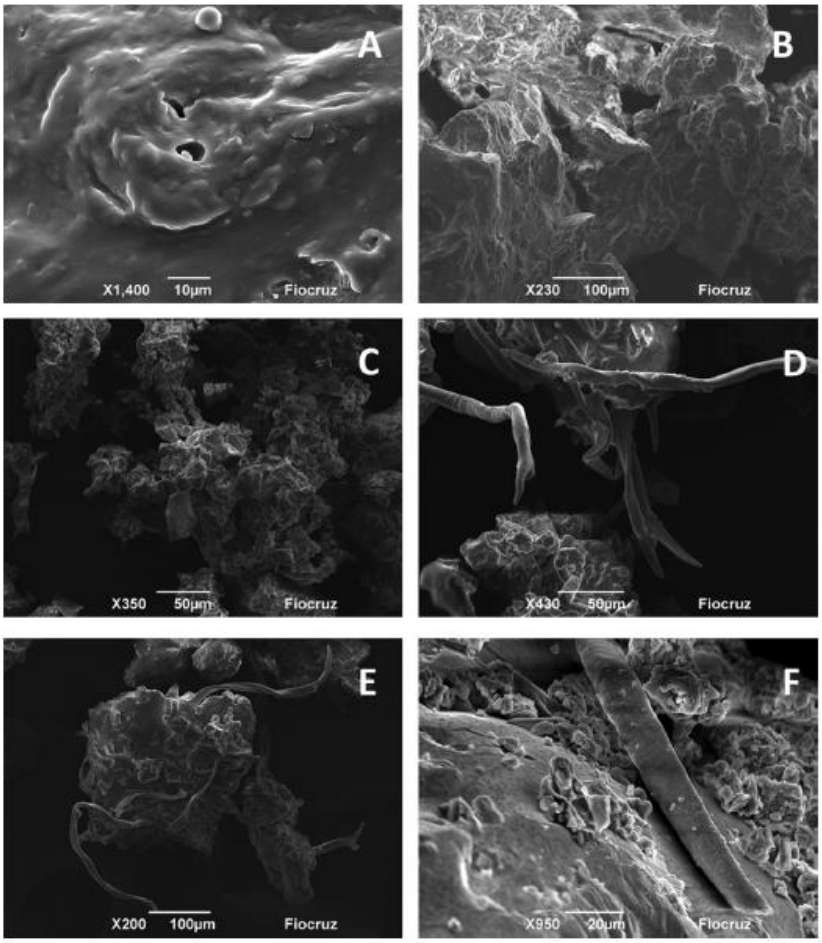

Fig 2. Images obtained by Scanning electron microscopy (SEM) for propolis samples. A–RSE; B–RAL; C–GMG_1; D–GMG_2; E–GPR; F–BSC; G–BPR; H–BRS.

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This confirms the biological potential of this "new" type of propolis [4,74–76]. The highest quantity of total phenols, flavonoids and the best antioxidant activity by ABTS was identified in the extract of red propolis from the state of Sergipe (SER EtOH), with values of 300.36±0.01 mg EAG/g, 57.60±0.01 mg EQ/g and 98.50±1.40%, respectively (Table 4). However, the best results for the antioxidant activity by DPPH was shown on the extract of green propolis from the state of Minas Gerais–GMG_1 EtOH (IC_{50} of 31.80±0.16).

These results indicate that the total concentration of phenolic compounds or flavonoids is not the only factor responsible for antioxidant properties. The chemical nature of the phenolic compounds and, perhaps, the presence of other compounds contribute to the total antioxidant capacity of the extracts [77]. The extracts EtOH and SCO_2 obtained from brown propolis showed the lowest values of phenols, flavonoids and antioxidant activity, therefore showing the lowest biological potential of this type of propolis when compared to the samples of green and/or red Brazilian propolis evaluated in the study.

Table 4. Determination of the content of total phenolic compounds (mg EAG/g), flavonoids (mg EQ/g), antioxidant activity by DPPH (IC_{50}) and ABTS (%) of the extracts of different samples from Brazilian propolis obtained by ethanolic extraction (EtOH) and by SFE (SCO_2).

Samples	Phenolic Compounds(mg EAG/g)	Flavonoids(mg EQ/g)	DPPH(IC_{50})	ABTS (%)(Trolox 1 mg.ml^{-1})
RSE EtOH	300.36±0.01^{a}	57.60±0.01^{a}	89.32±0.28^{a}	98.50±1.40^{a}
RSE SCO_2	157.43±0.01^{b}	25.46±0.01^{b}	116.49±0.23^{b}	87.60±7.20^{b,d}
RAL EtOH	198.77±0.01^{c}	58.19±0.01^{c}	44.29±0.29^{c}	98.20±1.30^{a}
RAL SCO_2	157.16±0.01^{b}	40.65±0.01^{d}	183.11±0.31^{d}	82.80±3.50^{b}
GMG_1 EtOH	181.71±0.01^{d}	46.80±0.01^{e}	31.80±0.16^{e}	77.90±6.80^{c}
GMG_1 SCO_2	137.52±0.01^{e}	25.02±0.01^{b}	97.74±0.22^{f}	76.70±1.29^{c}
GMG_2 EtOH	160.98±0.01^{f}	25.52±0.01^{b}	101.45±0.23^{g}	86.40±2.48^{b}
GMG_2 SCO_2	111.33±0.01^{g}	24.52±0.01^{f}	93.02±0.20^{h}	77.60±1.39^{c}
GPR EtOH	179.52±0.01^{h}	39.90±0.01^{g}	157.39±0.26^{i}	89.90±1.80^{d}
GPR SCO_2	118.14±0.03^{j}	29.71±0.01^{h}	85.34±0.23^{j}	73.80±1.80^{e}
BSC EtOH	117.03±0.01^{j}	27.97±0.01^{i}	163.00±0.31^{l}	89.80±1.20^{b,d}
BSC SCO_2	218.09±0.01^{l}	31.38±0.01^{j}	331.88±0.09^{m}	72.70±5.30^{f}
BRS EtOH	111.25±0.01^{g}	27.72±0.01^{i}	273.46±0.24^{n}	94.10±4.00^{g}
BRS SCO_2	172.43±0.01^{m}	29.72±0.01^{h,l}	306.91±0.09^{o}	76.80±1.10^{c}
BPR EtOH	110.92±0.01^{n}	24.40±0.01^{f}	164.52±0.34^{p}	81.90±1.73^{b}
BPR SCO_2	97.97±0.01^{o}	11.55±0.01^{m}	373.53±0.15^{q}	49.60±4.10^{h}

Values showing the same letter on the same column do not show significant difference ($p>0.05$) through the Tukey test at 95% confidence level. EtOH–Extracts obtained by ethanolic extraction; SCO_2 –Extracts obtained by SFE (CO_2 as supercritical fluid); Lower values of IC_{50} indicate higher activity of radical elimination.

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Frozza et al. [32] identified a content of 151.55±1.95 mg/g of phenolic compounds and a IC_{50} of 270.13±24.77, whereas Alencar et al. [18] found a content of 232.00±22.30 mg/g for total phenols, 43.00±1.00 for flavonoids and a IC_{50} of 57.00±3.20, for ethanolic extracts of red propolis from Sergipe and Alagoas (Brazil), respectively. Cottica et al. [77] determined values

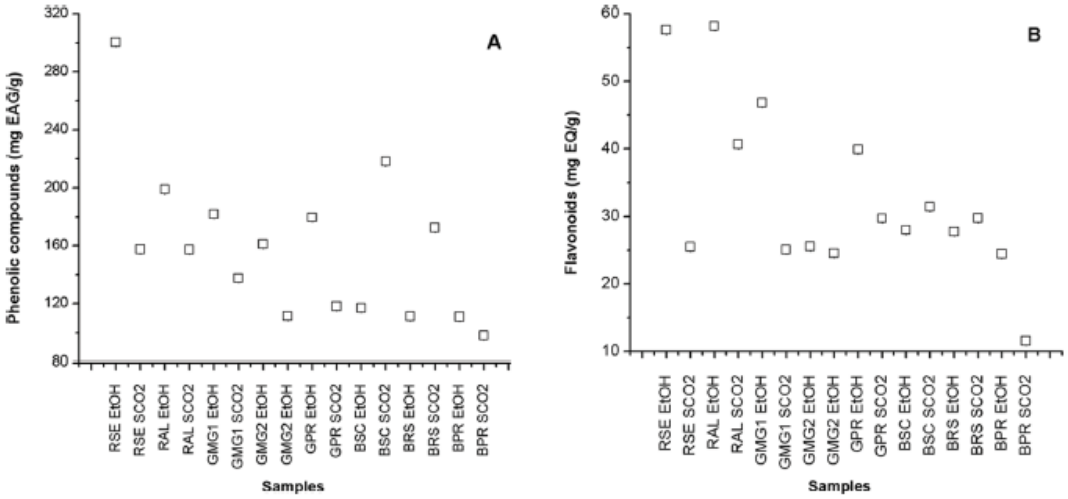

Fig 3. Content of total phenolic compounds expressed in mg EAG/g (A) and of flavonoids expressed in mg EQ/G (B) of the extracts of different samples of Brazilian propolis. SCO_2 –Extracts obtained by SFE; EtOH–Extracts obtained by ethanolic extraction; Lower values of IC_{50} indicate a higher activity of radical elimination; Average of analysis obtained in triplicate (n = 3).

doi:10.1371/journal.pone.0145954.g003

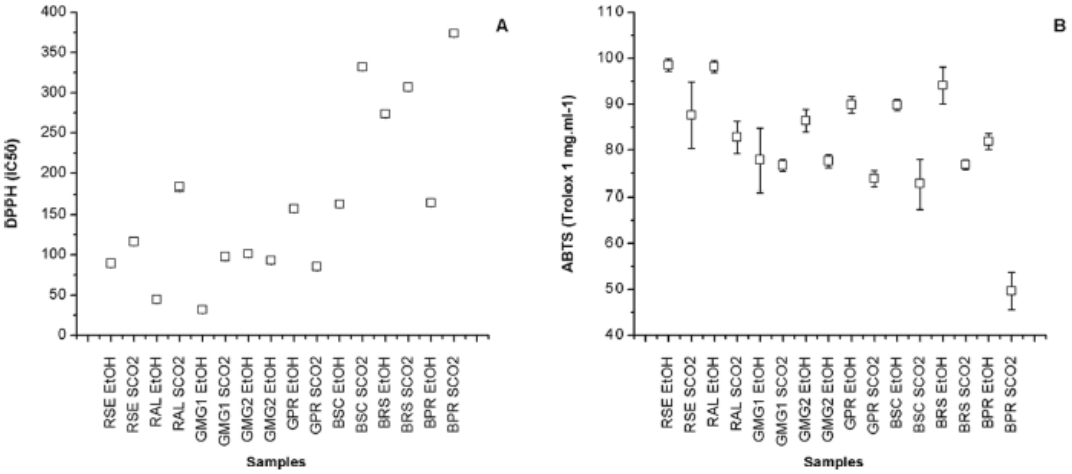

Fig 4. Determination of antioxidant activity of the extracts from different samples of Brazilian propolis, by DPPH (IC_{50}) (A) and ABTS (Trolox 1 mg.ml^{-1}) (B). SCO_2 –Extracts obtained by SFE; EtOH–Extracts obtained by ethanolic extraction; Average of analysis obtained in triplicate (n = 3).

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of DPPH (IC_{50}) among 47 and 160 ng/ml in extracts of hydro alcohols from the Brazilian green propolis, whereas Christov et al. [78] found values between 65 and 79% of inhibition by DPPH for ethanolic extract of Canadian propolis at 210 ng/ml. The results identified in this study are in accordance with the literature [79].

Significant differences were identified ($p>0.05$) for the results of the compounds analysed (Table 4), when compared to the extraction method for the same sample, as well as for the extracts obtained through the same method and samples of different types. The variations identified among the samples ($p>0.05$) were already expected, considering that the propolis obtained from different phytogeography regions exhibit very distinct chemical profiles [80–81]. The results found in this study confirm the influence of the origin of the raw material on the composition and characteristics of the extracts.

Chaillou and Nazareno [82] also observed significant ($p>0.05$) differences on the content of phenolic compounds, flavonoids and antioxidant activity, when evaluating different samples of propolis originated from Santiago del Estero, Argentina, where the averages varied from 92 to 187 mg/g for polyphenols, from 6 to 18 mg/g for flavonoids, and from 49.5 to 65.7% for DPPH. Similar results were also identified by Kumazawa et al. [83], by Kalogeropoulos et al. [84] and Choi et al. [85], when propolis from different geographic origins were evaluated. It stands out that the variability found between the content of compounds and antioxidant activities of propolis from Brazil is attributed to the differences observed in the arboreal species for each geographic area, being justified by the great Brazilian diversity.

Comparatively evaluating the results obtained for the extracts EtOH and SCO_2 of the same sample, it is noted that the majority of extracts EtOH present the best results for the content of total phenolic compounds and flavonoids, with the exception of the samples BSC and BRS, where the extracts SCO_2 shows superior values of these compounds. Generally, the extracts obtained by ethanolic extraction (EtOH) show the best antioxidant activities through the methods DPPH and ABTS.

Similar results were observed by Miguel et al. [86] and Cottica et al. [77], who found higher values of total phenols and flavonoids in EtOH extracts of Portuguese and Canadian propolis, respectively, in relation to the aqueous extracts. Zordi et al. [87] also identified higher

concentrations in EtOH extracts of Italian propolis when compared to the extracts obtained by SFE in different conditions of process and using CO_2. Lee et al. [1] also verified that the extracts of Brazilian propolis obtained by SFE showed a lower antioxidant capacity (DPPH) when compared to the extracts obtained by Soxhlet, hot extraction and by ultrasound. However, Laskar et al. [88] reported that the phenolic compounds in aqueous extracts of Indian propolis were in higher concentration when compared to EtOH extracts.

The lower concentrations of phenolic compounds, flavonoids and antioxidant activity identified in the extracts obtained by SFE (SCO_2) confirms the higher presence of undesirable substances such as wax, resin and other materials present in propolis, which could indicate a lower biological potential for these extracts [24]. Wax and other organic detritus are removed during the process of ethanolic extraction, and the propolis extracts obtained that way can contain the majority of the antioxidant constituents [84].

Zordi et al., [87] indicated two possible applications for the use of supercritical CO_2 for samples of propolis: obtain lipophilic fractions enriched by specific components, or as a pre-treatment of the raw material to facilitate the additional extraction with ethanol. As described by Biscaia and Ferreira [24], complex natural matrices such as propolis, can result in different products, depending on the method used. Therefore, the viability of the process is related to the yield and quality of the product, in order to improve the biological potential present in the raw material. With that, the efficacy of the extraction method (higher or lower selectivity) for obtaining total phenolic compounds and flavonoids can vary according to the origin and composition of the raw material.

Quantification of Artepillin C and p-coumaric acid in EtOH and SCO_2 extracts

The results of the quantitative analysis of Artepillin C and p-coumaric acid on the extracts of the different samples of Brazilian propolis are shown on Table 5. As expected, the markers Artepillin C and p-coumaric acid were present in all extracts of green propolis [1,7,10,89–91]. The Fig 5 shows the chromatogram of a green propolis samples obtained by ethanolic extraction (GPR EtOH). Artepillin C was also identified in two samples of brown propolis originated from the regions of Santa Catarina (SC) and Paraná (PR). As described by Lee et all. [1], different types of propolis can contain a varied quantity of Artepillin C, however the green propolis, originated from the vegetal species *Baccharis dracunculifolia* shows a higher quantity of this compound.

The content of p-coumaric acid varied from 5.05±0.10 (BSC SCO_2) to 198.09±3.12 μg/ml (GPR SCO_2), whereas that of the Artepillin C varied from 58.32±1.00 (BSC EtOH) to 845.05 ±0.12 μg/ml (GPR EtOH) among the extracts. The results show differences among the samples ($p>0.05$), which are in conformity with its place of origin. The green propolis from Paraná (PR) was the one which presented the highest values of the studied compounds. Kumazawa et al. [83] identified the presence of p-coumaric acid and Artepillin C (43.9 mg/g) in propolis from Brazil when evaluating samples from different countries.

It was also identified that, from the 16 samples analysed, the Artepillin C was only present on the green propolis from Brazil. Tazawa et al. [92] also concluded that the p-coumaric acid and Artepillin C are the main active components of the Brazilian propolis, whereas certain flavonoids are the main constituents of propolis from other countries (China, Japan and Bulgaria, among others). Shimizu et al. [93] identified a high quantity of Artepillin C (21.0 mmol/100g) and p-coumaric acid (7.70 mmol/100g) in the Brazilian propolis from Minas Gerais. Ahn et al. [94] evaluated samples of propolis collected from different regions of China and observed the presence of p-coumaric acid in all samples, which varied from 2.3 to 42.3 mg/g.

Table 5. Determination of the content of 4-hidroxicinamic acid (p-coumaric acid) and 3,5-diprenil-4-hidroxicinamic (Artepillin C) of extracts from different samples of Brazilian samples obtained by ethanolic extraction (EtOH) and by SFE (SCO_2).

Samples	p-coumaric acid (μg/ml)	Artepillin C (μg/ml)
RSE EtOH	----	----
RSE SCO_2	----	----
RAL EtOH	----	----
RAL SCO_2	----	----
GMG1 EtOH	24.65±0.24[a]	569.85±0.11[a]
GMG1 SCO_2	195.12±6.12[b]	798.05±1.20[b]
GMG2 EtOH	26.64±1.56[a]	340.89±1.11[c]
GMG2 SCO_2	101.68±2.87[c]	539.22±2.23[d]
GPR EtOH	35.57±3.45[d]	464.49±9.23[e]
GPR SCO_2	198.09±3.12[e]	845.05±0.12[f]
BSC EtOH	----	58.32±1.00[g]
BSC SCO_2	5.05±0.10[f]	106.81±1.08[h]
BRS EtOH	----	----
BRS SCO_2	----	----
BPR EtOH	----	82.67±6.12[i]
BPR SCO_2	----	315.96±5.89[j]

Values showing the same letter, in the same column, do not show significant differences (p>0.05) by the Tukey test at 95% confidence interval; EtOH–Extracts obtained by ethanolic extraction; SCO_2 –Extracts obtained by SFE (CO_2 as supercritical fluid); ----Not identified.

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It becomes important to note that the extraction with supercritical CO_2 (SCO_2) was significantly more efficient for obtaining both analysed markers, when compared to the same sample ($p>0.05$). The extracts obtained by SFE can have a concentration four times higher than that

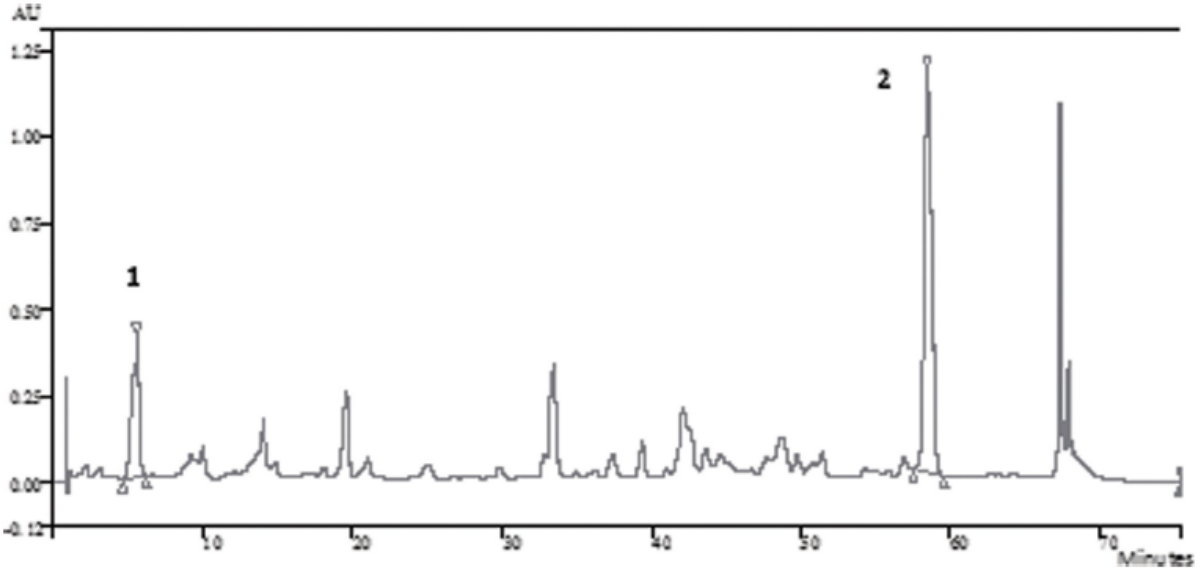

Fig 5. Chromatograms of green propolis ethanolic extract from Paraná (GPR EtOH)–(1) p-coumaric acid; (2) Artepillin C.

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of the p-coumaric acid, when compared to the EtOH extracts. Generally, despite this not being the most efficient method for obtaining the total phenolic compounds and antioxidant activity of the samples, it is clear that it provides a higher selectivity to obtain both analysed compounds, Artepillin C and p-coumaric acid in samples of Brazilian green propolis.

It is important to highlight that one of the main aspects that should be considered in SFE is the choice of operational conditions in the process of extraction, because that it can provide an additional advantage to the conventional methods, considering the lowest total yield and the highest cost. Besides that, the use of the optimized values for the different conditions can significantly improve the yield and the recovery of the target compound [30].

In this work, the optimized conditions of temperature, pressure, percentage of co-solvent and quantity of sample of a previous work from our group [43], which specifically evaluated the obtainment of Artepillin C and p-coumaric acid. In view of that, a better extraction of both phenolic acids by SFE is justified, when compared to EtOH extraction.

Similar results were identified by other authors, when the extraction of relevant compounds from different natural matrices using SFE and conventional extraction were compared. Those studies have shown the selectivity of SFE, generating products with higher biological value [31,95–99].

For example, Lee et al. [1] individually investigated organic solvents (conventional methods) and supercritical CO_2 to recover Artepillin C from the Brazilian propolis, and identified that the extracts obtained by SFE (45.3±0.10 mg/ml) showed the highest contents of the relevant compound (Soxhlet = 16.9±0.2 0mg/ml; Hot extraction = 16.4±0.23 mg/ml; Ultrasound = 16.0 ±0.06 mg/ml). Sun et al. [100] extracted the active substance paeonol from *Cynanchum paniculatum* by SFE and other conventional techniques, identifying 72.02% of the active substance in the extracts obtained by SFE, a highly superior result to that found by other methods of extraction (ultrasound 1.56%, steam distillation 1.64% and Soxhlet 2.74%).

The patent CN 1258511 (Chinese) and BR 1020140320121 (Brazilian) describe the extraction of active compounds from propolis by SFE. There, it is shown that the extracts obtained using CO_2 as supercritical fluid (and ethanol as co-solvent) are rich in different compounds (phenolic acids, flavones and terpenes) [26,101], being, therefore an efficient method for yield and selectivity for the extractive process of relevant compounds from propolis. Extracts of propolis obtained with supercritical fluids are already being sold in the markets of Japan, considering the proof of anti-tumour properties of these extracts [102].

Determination of the antimicrobial activity of the extracts EtOH and SCO_2

Table 6 shows the values of MIC and MBC obtained for the different SCO_2 and EtOH extracts of the propolis samples tested. It was noted that all extracts showed activity against gram-positive bacteria *Staphylococcus aureus* (ATCC 33951 and 25923) and gram-negative *Escherichia coli* (ATCC 25922), but this effect was dependent on the origin of the matrix and method of extraction. The control sample did not affect the growth of tested bacteria (data not shown). As expected, the extracts from different samples of propolis showed a higher activity against the gram-positive strains than against the gram-negative strains. These results are in accordance with those from Koru et al. [103], Vardar-Ünlü et al. [104], Kim and Chung [105] and Silva et al. [106], which can easily be explained by the structural differences of the bacterial cellular wall [107–108].

When compared to the extraction method, the EtOH extracts showed the best antimicrobial activities, and as previously shown, these extracts also had the best antioxidant activities and the highest content of total phenolic acids and flavonoids. Propolis samples from different

Table 6. Determination of Minimal Inhibitory Concentration (MIC) and Minimal Bactericide Concentration (MBC) of the extracts from different samples of Brazilian propolis obtained by ethanolic extraction (EtOH) and by SFE (SCO_2).

	Staphylococcus aureusATCC 25923		*Staphylococcus aureus ATCC 33591*		*Escherichia coli* ATCC 25922	
Samples	CIM (μg/mL)	CBM (μg/mL)	CIM (μg/mL)	CBM (μg/mL)	CIM (μg/mL)	CBM (μg/mL)
RSE EtOH	50–25	800–400	400–100	1600–800	400	1600–800
RSE SCO_2	100–50	800	600–200	1600	800	1600
RAL EtOH	100–50	800–400	400–100	1600–800	400	1600
RAL SCO_2	200–400	1600–800	800–400	1600	800	1600
GMG_1 EtOH	200	800	1600–50	>1600	1600–400	>1600
GMG_1 SCO_2	400	1600	1600–400	>1600	1600–800	>1600
GMG_2 EtOH	400–200	1600–800	800–200	1600	1600–800	>1600
GMG_2 SCO_2	800–400	1600	800–400	>1600	1600	>1600
GPR EtOH	400–200	1600–800	800–200	1600	1600–800	>1600
GPR SCO_2	800–400	1600	800	>1600	1600	>1600
BSC EtOH	800–400	1600–800	>1600	>1600	1600–800	>1600
BSC SCO_2	800	1600	>1600	>1600	1600	>1600
BRS EtOH	800–400	>1600	>1600	>1600	1600–800	>1600
BRS SCO_2	1600–800	>1600	>1600	>1600	1600	>1600
BPR EtOH	400–200	1600–800	800–200	1600	1600–800	>1600
BPR SCO_2	800–400	1600	1600–800	>1600	1600	>1600

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regions of Europe and the Middle East were evaluated in a study performed by Popova et al. [109] and a negative correlation between the concentration of phenolics in the extract and MIC was identified. The test was performed with hydro alcoholic extracts of propolis against *Staphylococcus aureus*, and it was found that the higher concentration of phenolics, the more powerful the activity against this bacteria. Jug et al. [110] also evaluated the antibacterial and antifungal efficiency of propolis extracts obtained by different extraction techniques and identified that the EtOH extract showed the best antimicrobial potential.

The extract that showed the highest antimicrobial activity *in vitro* for the three tested strains was the RSE EtOH, which also showed the highest content of phenolic compounds and high values of flavonoids. Among the samples evaluated, the extracts obtained from the samples of red propolis showed the best antimicrobial activities. Koo et al. [55] evaluated extracts of propolis from different types and regions of Brazil (red propolis from Bahia and green propolis from Minas Gerais and Paraná), identified differences in the MIC and MBC for each extract in relation to *Streptococcus mutans*, *S. sobrinus* and *S. cricetus*, and also that the best results were shown by the red propolis from the Brazilian northeast, as identified in this study.

Alencar et al. [18] also identified a noticeable antimicrobial activity for ethanolic and chloroformic extracts of Brazilian red propolis (Alagoas) against the *Staphylococcus aureus* ATCC 25923 (MIC of 50–100 and MBC of 200–400 –EtOH extract; MIC of 200–400 and MBC of 100–200 –chloroformic extracts) and *Staphylococcus mutans* UA159. It were concluded that the best antimicrobial activity was found for the extract with the highest concentration of chloroformic total phenols. For the extracts from green propolis, the samples GMG_1 and GPR showed the best antimicrobial potential. The extracts of brown propolis, which showed the lowest antioxidant potentials, presented the highest concentrations for the inhibition of antimicrobial growth for the strains tested. As expected and identified in other studies [18,109,111], the MBC for all extracts was four times superior to the MIC.

As already described in other studies, and also identified in this work, regardless of its geographic origin, propolis shows an important antimicrobial activity, since this property is essential for the preservation and maintenance of the hive [84,103,112–114]. Different studies show that the antimicrobial activity of this matrix is mainly due to complex synergic effects between the flavonoids, phenolic acids and its derivatives, which are mainly present in propolis [39,115–116].

For example, when the propolis extracts originated from Brazil and Bulgaria were evaluated against strains of *Staphylococcus aureus*, it was found that the Brazilian extracts showed the best antimicrobial potential, which had the highest concentration of phenolic compounds [117]. Although the mechanism of action for the antimicrobial effect of propolis is still not clearly understood and defined, some studies suggest that certain constituents can interfere in the process of bacterial cell division through disorganizing the cytoplasm, causing cellular lysis [118–119]. It was also found in this study, as previously reported by other authors, that the antimicrobial activity of propolis extracts is related to the method of extraction and type of solvent used [110,120]. Therefore, the determination of MIC and MBC is extremely important to evaluate the quality of the extracts and propolis-based products [103,105,108], considering the great variability in its composition.

Determination of antitumoral activity *in vitro* of EtOH extracts

The present study also investigated the antitumoral activity of the EtOH extracts of the eight samples of propolis against the cellular strains of melanoma murine (B16F10), evaluating the anti-proliferative effects. Generally, the ethanol extracts showed the best results for content of phenolic compounds, flavonoids and antioxidant activity (DPPH and ABTS). Because of that, those extracts were selected for analysis *in vitro* against human cancer cell lines B16F10. Moreover, the cost associated with these tests do not enables the evaluation of extracts obtained by supercritical fluid extraction. Therefore, comparative and statistical analysis was performed only between different samples of propolis, considering only an ethanol extraction method.

On Fig 6A and 6B are shown the activity on the cellular proliferation of the strain B16F10 after 24 and 48 hours of incubation on both concentrations tested (50 and 100 μg/ml). After 24

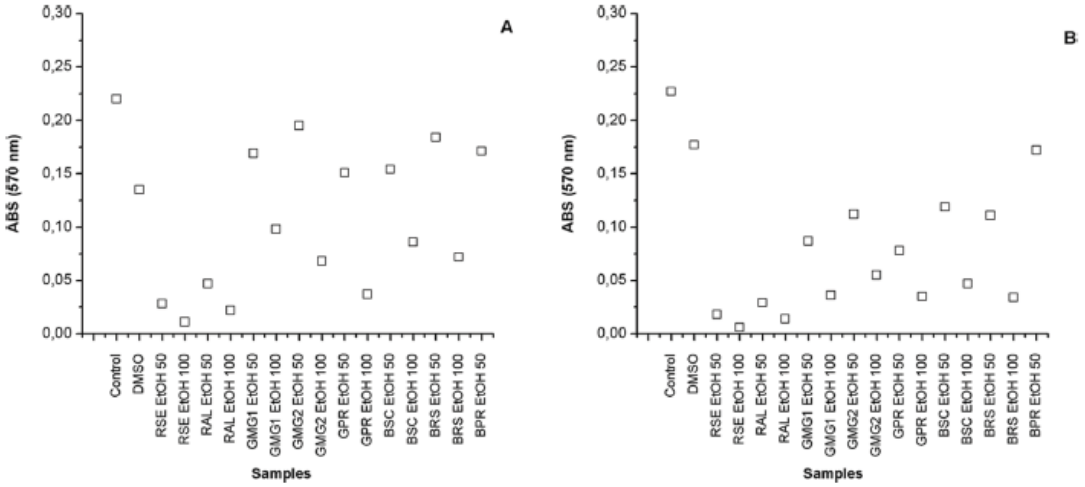

Fig 6. Activity of the EtOH extracts of different samples of Brazilian propolis on the cellular proliferation of the strain B16F10 (murine) after 24 (A) and 48 (B) hours of incubation on both concentrations tested (50 and 100 μg/ml).

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and 48 hours of incubation, all extracts showed a significant inhibition of cellular proliferation in comparison to the control ($p>0.05$).

The best results were shown by the extracts derived from the red propolis from the north-east region of Brazil, that is, Sergipe (RSE EtOH) and Alagoas (RAL EtOH). After 48 hours of incubation, the extracts RSE EtOH with concentrations of 50 and 100 μg/ml showed the lowest contents of viable cells, showing absorbance values of 0.018±0.002 and 0.006±0.001 ($1x10^5$ cells/ml), respectively. Among the green propolis extracts studied, the sample originated from Paraná, in Brazil (GPR EtOH) showed the best results for proliferative inhibition of cells B16F10. This extract also showed the highest concentration of Artepillin C and p-coumaric acid (Table 6).

The extracts showing the lower potential were those obtained from the brown propolis. The results found for antitumoral activity are in accordance to those previously identified for the content of antioxidant compounds (Table 5). Similar results to this study were found by Franchi-Jr et al. [121], when identifying that the *in vitro* cytotoxic activity of ethanolic extracts of red propolis against strains of human leukemic cells was superior when compared to the extracts of green propolis. Popolo et al. [122] also identified anti-proliferative effects of the ethanolic extract of the brown propolis from Cuba in cellular lineages of human breast cancer.

The best anti-proliferative effect showed by the red propolis extract, when compared to other samples of propolis may depend on its differentiated composition, for example, the presence of formononetin, the main isoflavones found in this type of propolis [123–124]. Recently, a polyisoprenylated benzophenone (xanthochymol) was also identified in the red propolis [123]. Different studies point to xanthochymol and formononetin as showing activity against tumoral cells [125–128].

It is likely that the anti-proliferative activity of this type of propolis occurs through the mechanism of cellular circle halt and apoptosis, as indicated in some studies [121,129–130]. Novak et al. [56] identified that an active fraction of the ethanolic extract of a sample of the Brazilian red propolis (João Pessoa) containing xanthochymol and formononetin showed superior anti-proliferative effects in strains of B16F10 cells, when compared only with the ethanolic extract of the sample.

In previous studies with the red propolis of the same geographic origin (Sergipe–RSE) different antitumoral effects against strains of bladder cancer cells and the presence of formononetin in its composition have been reported [76], and on larynx, uterus and kidney cancer cells [32]. López et al. [125] evaluated samples of red propolis from different regions and identified the presence of formononetin in every sample analysed, among which, four samples were from the state of Sergipe and two from Alagoas (Brazil).

Despite the fact that the green propolis, especially the GPR EtOH, show a lower potential when compared to the extract of red propolis (RSE and RAL), the inhibition in the presence of B16F10 cells was also very significant and relevant (Fig 5), especially when compared to the control ($p>0.05$). The presence of Artepillin C, a substance found in the Brazilian propolis, is the main constituent of the extracts of green propolis, followed by p-coumaric acid [83,94,131] and also attested by this work.

Carvalho et al. [132] also identified the anti-tumoral effects *in vitro* of the ethanolic and oily extracts of the green propolis from Paraná (Brazil), against different strains of human tumoral cells: HL-60 (leukaemia), HCT-8 (colon), MDA/MB-435 (breast) and SF-295 (brain). Kimoto et al. [133] identified that Artepillin C showed powerful cytocidal effects and induced levels of apoptosis in all the cellular lines of human leukaemia of different phenotypes evaluated. Kimoto et al. [89] identified that Artepillin C from Brazilian propolis showed cytotoxic effects and inhibited the growth of malign murine tumoral cells (B16F10) *in vitro* and *in vivo*, and that the mechanism of action is through the activation of the immunological system.

Matsuno et al. [134] reported that the Artepillin C is one of the most effective anti-tumoral compounds in green propolis, and Kimoto et al. [135] showed that this compound is capable of reducing the tumoral load in certain animal models. Akao et al [136] identified that the anti-tumoral inhibitory effects of the p-coumaric acid were less powerful than those of Artepillin C, and that the compounds available induced apoptosis in the cells characterized by nucleosome and DNA fragmentation analysis. Due to these biological properties, the propolis containing Artepillin C is considered a high quality propolis and the concentration of this component is already been used for the quality control in certain companies [67,132]. From the results identified, and together with other studies, the Brazilian propolis, especially the red and green, can be considered as an important source of active compounds for the development of new drugs with anti-tumoral potential.

Conclusions

In this study, it was determined that the total quantity of phenolic compounds, flavonoids and antioxidant activity are important parameters to evaluate the quality and biological potential of extracts from the Brazilian propolis, especially considering the great Brazilian biodiversity. The results identified significant differences among the samples ($p>0.05$), which are in conformity with their place of origin. Despite this chemical diversity, all the types of propolis showed a significant antimicrobial activity, and in most cases, it can be assumed that the compounds responsible are the phenolic constituents of propolis. In relation to the extraction method, generally, the ethanolic extraction (EtOH) was the most efficient for obtaining extracts with the highest content of antioxidant compounds and biological activity. However, the extraction with supercritical CO_2 (SCO_2) was the most efficient for obtaining Artepillin C and p-coumaric acid, evidencing the higher selectivity of SFE for obtaining both important markers for the Brazilian green propolis.

Finally, it must be noted that, due to its valuable properties and high biological potential, also already evidenced in other studies, the propolis can be considered as an important source of natural antioxidant compounds. New studies about the propolis complete chemical composition are under way.

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Author Contributions

Conceived and designed the experiments: BASM SSC OAD. Performed the experiments: BASM RPDS GAB DFS HNB JLCR. Analyzed the data: BASM JAPH MAUG FFP. Contributed reagents/materials/analysis tools: BASM HNB FFP JAPH OAD. Wrote the paper: BASM RPDS FFP HNB.

References

1. Lee YN, Chen CR, Yang HL, Lin CC, Chang CMJ. Isolation and purification of 3,5-diprenyl-4-hydroxycinnamic acid (artepillin C) in Brazilian propolis by supercritical fluid extractions. Sep Purif Technol. 2007; 54: 130–138. doi: 10.1016/j.seppur.2006.08.028

2. Toreti VC, Sato HH, Pastore GM, Park YK. Recent progress of propolis for its biological and chemical compositions and its botanical origin. Evid Based Complement Alternat Med. 2013; 2013: 697390. doi: 10.1155/2013/697390 PMID: 23737843. PMC:3657397.
3. Petelinc T, Polak T, Demšar L, Jamnik P. Fractionation of Phenolic Compounds Extracted from Propolis and Their Activity in the Yeast *Saccharomyces cerevisiae*. PLoS ONE. 2013; 8(2): e56104. doi: 10.1371/journal.pone.0056104 PMID: 23409133
4. Teles F, Silva TM, Cruz Júnior FP, Honorato VH, Costa OH, Barbosa APF, et al. Brazilian Red Propolis Attenuates Hypertension and Renal Damage in 5/6 Renal Ablation Model. PLoS ONE. 2015; 10: e0116535. doi: 10.1371/journal.pone.0116535 PMID: 25607548
5. Bankova VS, Castro SLD, Marcucci MC. Propolis: recent advances in chemistry and plant origin. Apidologie. 2000; 31: 3–15. doi: 10.1051/apido:2000102
6. Park YK, Alencar SM, Aguiar CL. Botanical origin and chemical composition of Brazilian propolis. J. Agric. Food Chem. 2002; 50(9): 2502–2506. doi: 10.1021/jf011432b PMID: 11958612
7. Hata T, Tazawa S, Ohta S, Rhyu MR, Misaka T, Ichihara K. Artepillin C, a Major Ingredient of Brazilian Propolis, Induces a Pungent Taste by Activating TRPA1 Channels. PLoS ONE. 2012; 7(11): e48072. doi: 10.1371/journal.pone.0048072 PMID: 23133611
8. Bankova V. Chemical diversity of propolis and the problem of standardization. J Ethnopharmacol. 2005; 100(1–2): 114–117. doi: 10.1016/j.jep.2005.05.004 PMID: 15993016.
9. Bankova VS, Popova M. Propolis of Stingless Bees: a Promising Source of Biologically Active Compounds. Pharmacogn Rev. 2007; 1: 88–92. Available: http://www.phcogrev.com/showBackIssue.asp?issn=09737847;year=2007;volume=1;issue=1;month=January-June.
10. Massaro FC, Brooks PR, Wallace HM, Nsengiyumva V, Narokai L, Russell FD. Effect of Australian Propolis from Stingless Bees (*Tetragonula carbonaria*) on Pre-Contracted Human and Porcine Isolated Arteries. PLoS ONE. 2013; 8(11): e81297. doi: 10.1371/journal.pone.0081297 PMID: 24260567
11. Castaldo S, Capasso F. Propolis, an old remedy used in modern medicine. Fitoterapia. 2002; 73: S1–S6. doi: 10.1016/S0367-326X(02)00185-5 PMID: 12495704
12. Funakoshi-Tago M, Okamoto K, Izumi R, Tago K, Yanagisawa K, Narukawa Y, et al. Anti-inflammatory activity of flavonoids in Nepalese propolis is attributed to inhibition of the IL-33 signaling pathway. Int. Immunopharmacol. 2015; 25: 189–198. doi: 10.1016/j.intimp.2015.01.012 PMID: 25614224
13. Teixeira EW, Negri G, Meira RMSA, Message D, Salatino A. Plant origin of green propolis: bee behavior, plant anatomy and chemistry. Evid Based Complement Alternat Med. 2005; 2: 85–92. doi: 10.1093/ecam/neh055 PMID: 15841282
14. Piantino CR, Aquino FWB, Follegatti-Romero LA, Cabral FA. Supercritical CO_2 extraction of phenolic compounds from *Baccharis dracunculifolia*. J. Supercrit Fluids. 2008; 47(2): 209–214. doi: 10.1016/j.supflu.2008.07.012
15. Daugsch A. The red propolis of northeast Brazil and its chemical and biological characteristics. D.Sc. Thesis, State University of Campinas. 2007. Available: http://www.bibliotecadigital.unicamp.br/document/?code=vtls000406573.
16. Silva BB, Rosalen PL, Cury JA, Ikegaki M, Souza VC, Esteves A, et al. Chemical composition and botanical origin of Red Propolis, a new type of Brazilian propolis. Evid Based Complement Alternat Med. 2008; 5(3): 313–316. doi: 10.1093/ecam/nem059 PMID: 18830449. PMCid:PMC2529384.
17. Falcão SI, Vale N, Gomes P, Domingues MRM, Freire C, Cardoso SM, et al. Phenolic profiling of Portuguese Propolis by LC–MS Spectrometry: Uncommon Propolis Rich in Flavonoid Glycosides. Phytochem Anal. 2013; 24(4): 309–318. doi: 10.1002/pca.2412 PMID: 23172843
18. Alencar SM, Oldoni TLC, Castro ML, Cabral ISR, Costa Neto CM, Cury JA, et al. Chemical composition and biological activity of a new type of Brazilian propolis: Red propolis. J Ethnopharmacol. 2007; 113(2): 278–283. doi: 10.1016/j.jep.2007.06.005 PMID: 17656055.
19. Barros MP, Sousa JPB, Bastos JK, Andrade SF. Effect of Brazilian green propolis on experimental gastric ulcers in rats. J Ethnopharmacol. 2007; 110(3): 567–571. doi: 10.1016/j.jep.2006.10.022 PMID: 17126509.
20. Ixtaina VY, Vega A, Nolasco SM, Tomás MC, Gimeno M, Bárzana E, et al. Supercritical carbon dioxide extraction of oil from Mexican chia seed (*Salvia hispanica* L.): Characterization and process optimization. J Supercrit Fluids. 2010; 55: 192–199. doi: 10.1016/j.supflu.2010.06.003
21. Uribe JAR, Perez JIN, Kauil HC, Rubio GR, Alcocer CG. Extraction of oil from chia seeds with supercritical CO_2. J Supercrit Fluids. 2011; 56(2): 174–178. doi: 10.1016/j.supflu.2010.12.007
22. Garmus TT, Paviani LC, Queiroga CL, Cabral FA. Extraction of phenolic compounds from pepper-rosmarin (*Lippia sidoides* Cham.) leaves by sequential extraction in fixed bed extractor using supercritical

CO_2, ethanol and water as solvents. J Supercrit Fluids. 2015; 99: 68–75. doi: 10.1016/j.supflu.2015.01.016

23. Pardo-Castaño C, Velásquez M, Bola-os G. Simple models for supercritical extraction of natural matter. J Supercrit Fluids. 2015; 97: 165–173. doi: 10.1016/j.supflu.2014.09.044
24. Biscaia D, Ferreira SRS. Propolis extracts obtained by low pressure methods and supercritical fluid extraction. J Supercrit Fluids. 2009; 51: 17–23. doi: 10.1016/j.supflu.2009.07.011
25. Chao-Rui C, Chun-Ting S, Jia-Jiuan W, Hsing-Ling Y, Shih-Lan H, Chieh-Ming JC. Precipitation of sub-micron particles of 3,5-diprenyl-4-hydroxycinnamic acid in Brazilian propolis from supercritical carbon dioxide anti-solvent solutions. J Supercrit Fluids. 2009; 50(2): 176–182. doi: 10.1016/j.supflu.2009.06.001
26. Machado BAS, Padilha FF, Nunes SB, Guedes CMC, Costa AS, Umsza-Guez MA, et al. Process for obtaining 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) propolis extract using supercritical carbon dioxide and co-solvent. 2014. Brazilian patent BR1020140320121.
27. Reverchon E, Marco I. Supercritical fluid extraction and fractionation of natural matter. J. Supercrit. Fluids. 2006; 38(2): 146–166. doi: 10.1016/j.supflu.2006.03.020
28. Mendiola JA, Herrero M, Cifuentes A, Ibáñes E. Use of compressed fluids for sample preparation: food applications. J Chromatogr. 2007; 1152(1–2): 234–246. doi: 10.1016/j.chroma.2007.02.046
29. Mattea F, Martín A, Cocero MJ. Carotenoid processing with supercritical fluids. J Food Eng. 2009; 93 (3): 255–265. doi: 10.1016/j.jfoodeng.2009.01.030
30. Machado BAS, Pereira CG, Nunes SB, Padilha FF, Umsza-Guez MA. Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives. Sep Sci Technol. 2013; 48(18): 2741–2760. doi: 10.1080/01496395.2013.811422
31. Wang BJ, Lien YH, Yu ZR. Supercritical fluid extractive fractionation-study of the antioxidant activities of propolis. Food Chem. 2004; 86(2): 237–243. doi: 10.1016/j.foodchem.2003.09.031
32. Frozza COS, Garcia CSC, Gambato G, Souza MDO, Salvador M, Moura S, et al. Chemical characterization, antioxidant and cytotoxic activities of Brazilian red propolis. Food Chem. Toxicol. 2013; 52: 137–142. doi: 10.1016/j.fct.2012.11.013 PMID: 23174518.
33. Ngatu NR, Saruta T, Hirota R, Eitoku M, Muzembo BA, Matsui T, et al. Antifungal efficacy of Brazilian green propolis extracts and honey on *Tinea capitis* and *Tinea versicolor*. Eur J Integr Med. 2011; 3(4): e281–e287. doi: 10.1016/j.eujim.2011.10.001
34. Fischer G, Conceição FR, Leite FPL, Dummer LA, Vargas GD'A, Hübner SO, et al. Immunomodulation produced by a green propolis extract on humoral and cellular responses of mice immunized with SuHV-1. Vaccine. 2007; 25(7): 1250–1256. doi: 10.1016/j.vaccine.2006.10.005 PMID: 17084001.
35. Cheung KW, Sze DM, Chan WK, Deng RX, Tu W, Chan GCF. Brazilian green propolis and its constituent, Artepillin C inhibits allogeneic activated human CD4 T cells expansion and activation. J Ethnopharmacol. 2011; 138(2): 463–471. doi: 10.1016/j.jep.2011.09.031 PMID: 21964192.
36. Barros MP, Lemos M, Maistro EL, Leite MF, Sousa JPB, Bastos JK, et al. Evaluation of antiulcer activity of the main phenolic acids found in Brazilian Green. J Ethnopharmacol. 2008; 120(3): 372–377. doi: 10.1016/j.jep.2008.09.015 PMID: 18930797.
37. Paulino N, Abreu SRL, Uto Y, Koyama D, Nagasawa H, Hori H, et al. Anti-inflammatory effects of a bioavailable compound, Artepillin C, in Brazilian propolis. Eur. J. Pharmacol. 2008; 587(1–3): 296–301. doi: 10.1016/j.ejphar.2008.02.067 PMID: 18474366.
38. Fernandes FH, Guterres ZR, Garcez WS, Lopes SM, Corsino J, Garcez. FR Assessment of the (anti) genotoxicity of brown propolis extracts from Brazilian Cerrado biome in a *Drosophila melanogaster* model. Food Res Int. 2014; 62: 20–26. doi: 10.1016/j.foodres.2014.02.029
39. Wilson MB, Brinkman D, Spivak M, Gardner G, Cohen JD. Regional variation in composition and antimicrobial activity of US propolis against *Paenibacillus larvae* and *Ascosphaera apis*. J. Invertebr. Pathol. 2015; 124: 44–50. doi: 10.1016/j.jip.2014.10.005 PMID: 25450740.
40. AOAC. Association of Official Analytical Chemists. Official methods of analysis of AOAC International. 16th ed. Washington: AOAC International; 1997.
41. Blight EG, Dyer WJ. A Rapid Method of Total Lipid Extraction and Purification. Can J Biochem Physiol. 1959; 37(8): 911–917. PMID: 13671378.
42. Van-Soest PJ, Wine RH. Use of detergents in analysis of fibrous feeds. In: Determination of plant cell wall constituents. J Assoc Off Anal Chem. 1967; 50: 50. Available: http://catalogo.latu.org.uy/doc_num.php?expInum_id=1418.
43. Machado BAS, Barreto GA, Costa AS, Costa SS, Silva RPD, Silva DF, et al. Determination of parameters for the supercritical extraction of antioxidant compounds from green propolis using carbon dioxide and ethanol as co-solvent. PLoS ONE. 2015; 10(8): e0134489. doi: 10.1371/journal.pone.0134489 PMID: 26252491

44. Brazil. Ministry of Health. National Health Surveillance Agency (ANVISA). Resolution n° 899, of may 29, 2003: Guide to the validation of analytical and bioanalytical methods. Available: http://portal.anvisa.gov.br/wps/wcm/connect/4983b0004745975da005f43fbc4c6735/RE_899_2003_Determina+a+publica%C3%A7%C3%A3o+do+Guia+para+valida%C3%A7%C3%A3o+de+m%C3%A9todos+anal%C3%ADticos+e+bioanal%C3%ADticos.pdf?MOD=AJPERES.

45. INMETRO. National Institute of Metrology, Standardization and Industrial Quality. Guidelines for Chemical Testing Methods Validation. 2011. Available: http://www.inmetro.gov.br/Sidoq/Arquivos/Cgcre/DOQ/DOQ-Cgcre-8_04.pdf.

46. Singleton VL, Rossi JA. Colorimetry of total phenolics with phosphomolybdic-phosphotungstic acid reagents. Am J Enol Vitic. 1965; 16(3): 144–158. Available: http://ajevonline.org/content/16/3/144.

47. Singleton VL, Orthofer R, Lamuela-Raventos RM. Analysis of total phenols and other oxidation substrates and antioxidants by means of FolinCiocalteu reagent. Meth Enzymol. 1999; 299: 152–178. doi: 10.1016/S0076-6879(99)99017-1

48. Peschel W, Sánchez-Rabaneda F, Diekmann W, Plescher A, Gartzia I, Jiménez D, et al. An industrial approach in the search of natural antioxidants from vegetable and fruit wastes. Food Chem. 2006; 97 (1):137–150. doi: 10.1016/j.foodchem.2005.03.033

49. Marcucci MC, Ferreres F, Guarcía-Viguera C, Bankova VS, Castro SL, Dantas AP, et al. Phenolic compounds from Brazilian propolis with pharmacological activities. J Ethnopharmacol. 2001; 74(2): 105–112. doi: 10.1016/S0378-8741(00)00326-3 PMID: 11167028.

50. Brand-William W, Cuvelier ME, Berset C. Use of free radical method to evaluate antioxidant activity. Lebenson Wiss Technol. 1995; 28: 25–30. doi: 10.1016/S0023-6438(95)80008-5

51. Molyneux P. The use of the stable free radical diphenylpicrylhydrazyl (DPPH) for estimating antioxidant activity. Songklanakarin J Sci Technol. 2004; 26: 211–219. Available: http://rdo.psu.ac.th/sjstweb/journal/26-2/07-DPPH.pdf.

52. Van-Den-Berg R, Haenen GRMM, Van-Den-Berg H, Bast A. Applicability of an improved Trolox equivalent antioxidant capacity (TEAC) assay for evaluation of antioxidant capacity measurements of mixtures. Food Chem. 1999; 66(4): 511–517. doi: 10.1016/S0308-8146(99)00089-8

53. Kim DO, Chun OK, Kim YJ, Moon HY, Lee CY. Quantification of polyphenolics and their antioxidant capacity in fresh plums. J Agric Food Chem. 2003; 51(22): 6509–6515. doi: 10.1021/jf0343074 PMID: 14558771.

54. NCCLS. National Committee for Clinical Laboratory Standards. Methods for dilution antimicrobial susceptibility tests for bacteria that grow aerobically (M100-S10 (M7)). Approved standard. 5ªed. Wayne, PA: NCCLS; 2000.

55. Koo H, Rosalen PL, Cury JA, Ambrosano GMB, Murata RM, Yatsuda R, et al. Effect of a New Variety of *Apis mellifera* Propolis on Mutants Streptococci. Curr Microbiol. 2000; 41(3): 192–196. doi: 10.1007/s0028400101170 PMID: 10915206.

56. Novak EM, Silva MSC, Marcucci MC, Sawaya ACHF, López BGC, Fortes MAZ, et al. Antitumoural activity of Brazilian red propolis fraction enriched with xanthochymol and formononetin: An *in vitro* and in vivo study. J Funct Foods. 2014; 11: 91–102. doi: 10.1016/j.jff.2014.09.008

57. Busatti HG, Gomes MA. A Simple Colourimetric Method to Determine AntiGiardial Activity of Drugs. Parasitol Res. 2007; 101(3): 819–821. doi: 10.1007/s00436-007-0525-8 PMID: 17387517.

58. Ozcan M, Ayar A. Effect of propolis extracts on butter stability. J Food Qual. 2003; 26: 65–73. doi: 10.1111/j.1745-4557.2003.tb00227.x

59. Narbona E, García-García E, Vazquez-Araújo L, Carbonell-Barrachina AA. Volatile composition of functional '*a la Piedra*'turrón with propolis. Int J Food Sci Technol. 2010; 45(3): 569–577. doi: 10.1111/j.1365-2621.2009.02167.x

60. Ali FH, Kassem GM, Atta-Alla OA. Propolis as a natural decontaminant and antioxidant in fresh oriental sausage. Vet Ital. 2010; 46(2): 167–172. PMID: 20560126.

61. Brazil. Ministry of Health. National Health Surveillance Agency (ANVISA). Normative Instruction n° 3, of January 19, 2001: Technical Regulations of identify and Quality of bee venom, royal Bee, Jelly Wax, Lyophilized Royal Jelly, Bee Pollen, Propolis and Propolis Extract. Available: http://extranet.agricultura.gov.br/sislegis-consulta/consultarLegislacao.do?operacao=visualizar&id=1798.

62. Bankova VS, Popova SS, Marekov NL, Maksimova V. The chemical composition of propolis fractions with antiviral action. Acta Microbiol Bulg. 1988; 23: 52–57. PMID: 3247872.

63. Marcucci MC, Rodriguez J, Ferrerez F, Bankova V, Groto R, Popov S. Chemical composition of Brazilian Propolis from São Paulo State. Z Naturforsch, C, Biosci. 1998; 53c: 117–119. Available: http://zfn.mpdl.mpg.de/data/Reihe_C/53/ZNC-1998-53c-0117.pdf.

64. Dias LG, Pereira AP, Estevinho LM. Comparative study of different Portuguese samples of propolis: Pollinic, sensorial, physicochemical, microbiological characterization and antibacterial activity. Food Chem Toxicol. 2012; 50(12): 4246–4253. doi: 10.1016/j.fct.2012.08.056 PMID: 22981908

65. Moreira TF. Chemical composition of propolis: vitamins and aminoacids. Braz Rev Farmacogn. 1986; 1(1): 12–19. doi: 10.1590/S0102-695X1986000100003

66. Bonvehi JS, Coll FV, Jordà RE. The composition, active components and bacteriostatic activity of propolis in dietetics. J Am Oil Chem Soc. 1994; 71(5): 529–532. doi: 10.1007/BF02540666

67. Funari CS, Ferro VO. Propolis analysis. Braz J Food Technol. 2006; 26: 171–178. doi: 10.1590/S0101-20612006000100028

68. Attia YA, Al-Hamid AEA, Ibrahim MS, Al-Harthi MA, Bovera F, Elnaggar ASh. Productive performance, biochemical and hematological traits of broiler chickens supplemented with propolis, bee pollen, and mannan oligosaccharides continuously or intermittently. Livest Sci. 2014; 164: 87–95. doi: 10.1016/j.livsci.2014.03.005

69. Tylkowski B, Trusheva B, Bankova V, Giamberini M, Peev G, Nikolova A. Extraction of biologically active compounds from propolis and concentration of extract by nanofiltration. J Memb Sci. 2010; 348(1–2): 124–130. doi: 10.1016/j.memsci.2009.10.049

70. Budel JM, Duarte MR, Santos CAM, Farago PV. Leaf and stem morpho-anatomy of *Baccharis dracunculifolia* DC., Asteraceae. Acta Farm Bonaerense. 2004; 23(4): 477–483. Available: http://www.latamjpharm.org/trabajos/23/4/LAJOP_23_4_1_8_I54P2Y1561.pdf.

71. Budel JM, Duarte MR. Pharmacobotanical study of aerial vegetative parts of *Baccharis anomala* DC., Asteraceae. Braz Rev Farmacogn. 2008; 18: 761–768. doi: 10.1590/S0102-695X2008000500022

72. Bastos EMAF, Santana RA, Calaça-Costa AGF, Thiago PS. Interaction between *Apis mellifera* L. and *Baccharis dracunculifolia* DC, that favours green propolis production in Minas Gerais. Braz J Biol. 2011; 71(3): 727–734. doi: 10.1590/S1519-69842011000400018 PMID: 21881797

73. Sawaya ACHF, Cunha IBS, Marcucci MC, Rodrigues RFD, Eberlin MN. Brazilian propolis of *Tetragonisca angustula* and *Apis mellifera*. Apidologie. 2006; 37(3): 398–407. doi: 10.1051/apido:2006011

74. Lotti C, Fernandez MC, Piccinelli AL, Cuesta-Rubio O, Hernandez IM, Rastrelli L. Chemical constituents of red Mexican propolis. J Agric Food Chem. 2010; 58(4): 2209–2213. doi: 10.1021/jf100070w PMID: 20121106.

75. Costa SS, Machado BAS, Souza CO, Druzian JI, Guimarães AG. Bi-Functional biobased packing of the cassava starch, glycerol, licuri nanocellulose and red propolis. Plos One. 2014; 9(11): e112554–e112554. doi: 10.1371/journal.pone.0112554 PMID: 25383783. PMCid:PMC4226550.

76. Begnini KR, Leon PMM, Thurow H, Schultze E, Campos V, Rodrigues FM, et al. Brazilian Red Propolis Induces Apoptosis-Like Cell Death and Decreases Migration Potential in Bladder Cancer Cells. Evid Based Complement Alternat Med. 2014; 2014: 1–13 doi: 10.1155/2014/639856 PMID: 25530785. PMCid:PMC4235187.

77. Cottica SM, Sabik H, Antoine C, Fortin J, Graveline N, Visentainer JV, et al. Characterization of Canadian propolis fractions obtained from two-step sequential extraction. Lebenson Wiss Technol. 2015; 60: 609–614. doi: 10.1016/j.lwt.2014.08.045

78. Christov R, Trusheva B, Popova M, Bankova V, Bertrand M. Chemical composition of propolis from Canada, its antiradical activity and plant origin. Nat Prod Res. 2005; 19:673–678. doi: 10.1080/14786410512331328159 PMID: 16076637

79. Nagai T, Inoue R, Inoue H, Suzuki N. Preparation and antioxidant properties of water extract of propolis. Food Chem. 2003; 80: 29–33. doi: 10.1016/S0308-8146(02)00231-5

80. Valencia D, Alday E, Robles-Zepeda R, Garibay-Escobar A, Galvez-Ruiz JC, Salas-Reyes M, et al. Seasonal effect on chemical composition and biological activities of Sonoran propolis. Food Chem. 2012; 131(2): 645–651. doi: 10.1016/j.foodchem.2011.08.086

81. Bankova V, Popova M, Trusheva B. Propolis volatile compounds: chemical diversity and biological activity: a review. Chem Cent J. 2014; 8:28. doi: 10.1186/1752-153X-8-28 PMID: 24812573

82. Chaillou LL, Nazareno MA. Bioactivity of propolis from Santiago del Estero, Argentina, related to their chemical composition. Lebenson Wiss Technol. 2009; 42(8): 1422–1427. doi: 10.1016/j.lwt.2009.03.002

83. Kumazawa S, Hamasaka T, Nakayama T. Antioxidant activity of propolis of various geographic origins. Food Chem. 2004; 84(3): 329–339. doi: 10.1016/S0308-8146(03)00216-4

84. Kalogeropoulos N, Konteles SJ, Troullidou E, Mourtzinos I, Karathanos VT. Chemical composition, antioxidant activity and antimicrobial properties of propolis extracts from Greece and Cyprus. Food Chem. 2009; 116(2): 452–461. doi: 10.1016/j.foodchem.2009.02.060

85. Choi YM, Noh DO, Cho SY, Suh HJ, Kim KM, Kim JM. Antioxidant and antimicrobial activities of propolis from several regions of Korea. Lebenson Wiss Technol. 2006; 39(7): 756–761. doi: 10.1016/j.lwt.2005.05.015

86. Miguel MG, Nunes S, Dandlen SA, Cavaco AM, Antunes MD. Phenols and antioxidant activity of hydro-alcoholic extracts of propolis from Algarve, South of Portugal. Food Chem. Toxicol. 2010; 48: 3418–3423. doi: 10.1016/j.fct.2010.09.014 PMID: 20849908.

87. Zordi N, Cortesi A, Kikic I, Moneghini M, Solinas D, Innocenti G, et al. The supercritical carbon dioxide extraction of polyphenols from propolis: a central composite design approach. J Supercrit Fluids. 2014; 95: 491–498. doi: 10.1016/j.supflu.2014.10.006

88. Laskar RA, Sk I, Roy N, Begum NA. Antioxidant activity of Indian propolis and its chemical constituents. Food Chem. 2010; 122: 233–237. doi: 10.1016/j.foodchem.2010.02.068

89. Kimoto T, Arai S, Kohguchi M, Aga M, Nomura Y, Micallef MJ, et al. Apoptosis and suppression of tumor growth by artepillin C extracted from Brazilian propolis. Cancer Detect Prev. 1998; 22(6): 506–515. doi: 10.1046/j.1525-1500.1998.00020.x PMID: 9824373.

90. Chen CR, Lee YN, Lee MR, Chang CMJ. Supercritical fluids extraction of cinnamic acid derivatives from Brazilian propolis and the effect on growth inhibition of colon cancer cells. J Taiwan Inst Chem Eng. 2009; 40(2): 130–135. doi: 10.1016/j.jtice.2008.07.014

91. Ikeda R, Yanagisawa M, Takahashi N, Kawada T, Kumazawa S, Yamaotsu N, et al. Brazilian propolis-derived components inhibit TNF-α-mediated downregulation of adiponectin expression via different mechanisms in 3T3-L1 adipocyte. Biochim Biophys Acta—General Subjects. 2011; 1810(7): 695–703. doi: 10.1016/j.bbagen.2011.04.007 PMID: 21554928.

92. Tazawa S, Warashina T, Noro T. On the chemical evaluation of propolis. J Nat Med (in Japanese). 2000; 54: 306–313. Available: http://chemport.cas.org/cgi-bin/sdcgi?APP=ftslink&action=reflink&origin=jstage2&version=1.0&coi=1%3ACAS%3A528%3ADC%252BD3MXhvVCjsLY%253D&md5=facc10806e7bfb2f89bd486b9076b58c.

93. Shimizu K, H Ashida H, Matsuura Y, Kanazawa K. Antioxidative bioavailability of Artepillin C in Brazilian propolis. Arch. Biochem. Biophys. 2004; 424(2): 181–188. doi: 10.1016/j.abb.2004.02.021 PMID: 15047190

94. Ahn MR, Kumazawa S, Usui Y, Nakamura J, Matsuka M, Zhu F, et al. Antioxidant activity and constituents of propolis collected in various areas of China. Food Chem. 2007; 101:1383–1392.

95. Chen CR, Shen CT, Wu JJ, Yang HL, Hsu SL, Chang CMJ. Precipitation of sub-micron particles of 3,5-diprenyl-4-hydroxycinnamic acid in Brazilian propolis from supercritical carbon dioxide anti-solvent solutions. J Supercrit Fluids. 2009; 50(2): 176–182. doi: 10.1016/j.supflu.2009.06.001

96. Donelian A, Carlson LHC, Lopes TJ, Machado RAF. Comparison of extraction of patchouli (Pogostemon cablin) essential oil with supercritical CO_2 and by steam distillation. J Supercrit Fluids. 2009; 48: 15–20. doi: 10.1016/j.supflu.2008.09.020

97. Glisic S, Ivanovic J, Ristic M, Skala D. Extraction of sage (*Salvia officinalis* L.) by supercritical CO_2: Kinetic data, chemical composition and selectivity of diterpenes. J Supercrit Fluids. 2010; 52: 62–70. doi: 10.1016/j.supflu.2009.11.009

98. Vargas CE, Mendes MF, Azevedo DA, Pessoa FLP, Uller AC. Extraction of the essential oil of abajeru (Chrysobalanus icaco) using supercritical CO_2. J Supercrit Fluids. 2010; 54(2): 171–177. doi: 10.1016/j.supflu.2009.12.007

99. Zhao S, Zhang D. Supercritical CO_2 extraction of Eucalyptus leaves oil and comparison with Soxhlet extraction and hydro-distillation methods. Sep Purif Technol. 2014; 133: 443–451. doi: 10.1016/j.seppur.2014.07.018

100. Sun Y, Liu Z, Wang J, Tian W, Zhou H, Zhu L, et al. Supercritical fluid extraction of paeonol from *Cynanchum paniculatum* (Bge.) Kitag. and subsequent isolation by high-speed counter-current chromatography coupled with high-performance liquid chromatography-photodiode array detector. Sep Purif Technol. 2008; 64(2): 221–226. doi: 10.1016/j.seppur.2008.10.007

101. Wu J, Luyu Q. Supercritical multiple extraction of bee glue. 2000. Chinese Patent, CN1258511.

102. Kimoto N, Hirose M, Kawabe M, Satoh T, Miyataka H, Shirai T. Post-initiation effects of a super critical extract of propolis in a rat two-stage carcinogenesis model in female F344 rats. Câncer Lett. 1999; 147(1–2): 221–227. doi: 10.1016/S0304-3835(99)00305-5 PMID: 10660110

103. Koru O, Toksoy F, Acikel CH, Tunca YM, Baysallar M, Guclu AU, et al. *In vitro* antimicrobial activity of propolis samples from different geographical origins against certain oral pathogens. Anaerobe. 2007; 13(3–4): 140–145. doi: 10.1016/j.anaerobe.2007.02.001 PMID: 17475517.

104. Vardar-Ünlü G, Silici S, Ünlü M. Composition and *in vitro* antimicrobial activity of Populus buds and poplar-type propolis. World J Microbiol Biotechnol. 2008; 24(7): 1011–1017. doi: 10.1007/s11274-007-9566-5

105. Kim YH, Chung HJ. The effects of Korean Propolis against foodborne pathogens and transmission electron microscopic examination. N Biotechnol. 2011; 28(6): 713–718. doi: 10.1016/j.nbt.2010.12.006 PMID: 21232643.

106. Silva JC, Rodrigues S, Feás X, Estevinho LM. Antimicrobial activity, phenolic profile and role in the inflammation of propolis. Food Chem Toxicol. 2012; 50(5): 1790–1795. doi: 10.1016/j.fct.2012.02.097 PMID: 22425940

107. Silici S, Kutluca S. Chemical composition and antibacterial activity of propolis collected by three different races of honeybees in the same region. J Ethnopharmacol. 2005; 99: 69–73. doi: 10.1016/j.jep.2005.01.046 PMID: 15848022.

108. Mohammadzadeh S, Shariatpanahi M, Hamedi M, Ahmadkhaniha R, Samadi N, Ostad SN. Chemical composition, oral toxicity and antimicrobial activity of Iranian propolis. Food Chem. 2007; 103(4): 1097–1103. doi: 10.1016/j.foodchem.2006.10.006

109. Popova MP, Bankova VS, Bogdanov S, Tsvetkova I, Naydenski C, Marcazzan GL, et al. Chemical characteristics of poplar type propolis of different geographic origin. Apidologie. 2007; 38(3): 306–311. doi: 10.1051/apido:2007013

110. Jug M, Končić MZ, Kosalec I. Modulation of antioxidant, chelating and antimicrobial activity of poplar chemo-type propolis by extraction procures. Lebenson Wiss Technol. 2014; 57(2): 530–537. doi: 10.1016/j.lwt.2014.02.006

111. Hayacibara MF, Koo H, Rosalen PL, Duarte S, Franco EM, Bowen WH, et al. *In vitro* and in vivo effects of isolated fractions of Brazilian propolis on caries development. J Ethnopharmacol. 2005; 101(1–3): 110–115. doi: 10.1016/j.jep.2005.04.001 PMID: 15913934.

112. Hegazi AG, Abd El Hady FK. Egyptian propolis: 3. Antioxidant, antimicrobial activities and chemical composition of propolis from reclaimed lands. Z Naturforsch C. 2002; 57(3–4): 395–402. doi: 10.1515/znc-2002-3-432 PMID: 12064746.

113. Ghasem YB, Ownagh A, Hasanloei M. Antibacterial and antifungal activity of Iranian propolis against Staphylococcus aureus and Candida albicans. Pak J Biol Sci. 2007; 10(8): 1343–1345. doi: 10.3923/pjbs.2007.1343.1345 PMID: 19069941.

114. Patel J, Ketkar S, Patil S, Fearnley J, Mahadik KR, Paradkar AR. Potentiating antimicrobial efficacy of propolis through niosomal-based system for administration. Integr Med Res. 2014. doi: 10.1016/j.imr.2014.10.004

115. Sawaya ACHF, Souza KS, Marcucci MC, Cunha IBS, Shimizu MT. Analysis of the composition of Brazilian propolis extracts by chromatography and evaluation of their *in vitro* activity against gram-positive bacteria. Braz J Microbiol. 2004; 35(1–2): 104–109. doi: 10.1590/S1517-83822004000100017

116. Melliou E, Stratis E, Chinou I. Volatile constituents of propolis from various regions of Greece–Antimicrobial activity. Food Chem. 2007; 103(2): 375–380. doi: 10.1016/j.foodchem.2006.07.033

117. Bankova V, Boudourova-Krasteva G, Popov S, Sforcin JM, Funari SRC. Seasonal variations of the chemical composition of Brazilian propolis. Apidologie. 1998; 29(4): 361–367. doi: 10.1051/apido:19980406

118. Takaisi-Kikuni NB, Schilcher H. Electron microscopic and microcalorimetric investigations of the possible mechanism of the antibacterial action of a defined propolis provenance. Planta Med. 1994; 60 (3): 222–227. doi: 10.1055/s-2006-959463 PMID: 8073087.

119. Tsuchiya H, Iinuma M. Reduction of membrane fluidity by antibacterial sophoraflavone G isolated from Sophora exigua. Phytomedicine. 2000; 7(2): 161–165. doi: 10.1016/S0944-7113(00)80089-6 PMID: 10839220

120. Buriol L, Finger D, Schmidt EM, Santos JMT, Rosa MR, Quináia SP, et al. Composição química e atividade biológica de extrato oleoso de própolis: uma alternativa ao extrato etanólico. Quím Nova. 2009; 32(2): 296–302. doi: 10.1590/S0100-40422009000200006

121. Franchi GC Jr, Moraes CS, Toreti VC, Daugsch A, Nowill AE, Park YK. Comparison of Effects of the Ethanolic Extracts of Brazilian Propolis on Human Leukemic Cells As Assessed with the MTT Assay. J Evid Based Complementary Altern Med. 2012; 2012: 1–6. doi: 10.1155/2012/918956

122. Popolo A, Piccinelli LA, Morello S, Cuesta-Rubio O, Sorrentino R, Rastrelli L, et al. Antiproliferative activity of brown Cuban propolis extract on human breast cancer cells. Nat Prod Comun. 2009; 4 (12):1711–1716. PMID: 20120113.

123. Piccinelli AL, Lotti C, Campone L, Cuesta-Rubio O, Campo Fernandez M, Rastrelli L. Cuban and Brazilian red propolis: botanical origin and comparative analysis by high-performance liquid chromatography-photodiode array detection/electrospray ionization tandem mass spectrometry. J Agric Food Chem. 2011; 22; 59(12):6484–91. doi: 10.1021/jf201280z PMID: 21598949.

124. Frozza COS, Ribeiro TS, Gambato G, Menti C, Moura S, Pinto PM, et al. Proteomic analysis identifies differentially expressed proteins after red propolis treatment in Hep-2 cells. Food Chem. Toxicol. 2014; 63: 195–204. doi: 10.1016/j.fct.2013.11.003 PMID: 24239894.

125. López BG, Schmidt EM, Eberlin MN, Sawaya AC. Phytochemical markers of different types of red propolis. Food Chem. 2014; 1(146):174–180. doi: 10.1016/j.foodchem.2013.09.063

126. Cuesta-Rubio O, Fontana-Uriba BA, Ramirez-Apan T, Cardenas J. Polyisoprenylated benzophenones in Cuban propolis: biological activity of nemorosone. Z Naturforsch. 2002; 57:372–378. doi: 10.1515/znc-2002-3-429

127. Ye Y, Hou R, Chen J, Mo L, Zhang J, Huang Y, et al. Formononetin-induced Apoptosis of Human Prostate Cancer Cells Through ERK1/2 Mitogen-activated Protein Kinase Inactivation. Horm Metab Res. 2012; 44(04): 263–267. doi: 10.1055/s-0032-1301922

128. Zhang X, Bi L, Ye Y, Chen J. Formononetin Induces Apoptosis in PC-3 Prostate Cancer Cells Through Enhancing the Bax/Bcl-2 Ratios and Regulating the p38/Akt Pathway. Nutr Cancer. 2014; 66(4): 656–661. doi: 10.1080/01635581.2014.894098 PMID: 24666255

129. Matsumoto K, Akao Y, Kobayashi E, Ito T, Ohguchi K, Tanaka T, et al. Cytotoxic benzophenone derivatives from *Garcinia* species display a strong apoptosis-inducing effect against human leukaemia cell lines. Biol. Pharm. Bull. 2003; 26(4): 569–571. doi: 10.1248/bpb.26.569 PMID: 12673047.

130. Chen J, Sun L. Formononetin-induced apoptosis by activation of Ras/p38 mitogen-activated protein kinase in estrogen receptor-positive human breast cancer cells. Horm Metab Res. 2012; 44(13): 943–948. doi: 10.1055/s-0032-1321818 PMID: 22828872.

131. Shimizu K, Ashida H, Matsuura Y, Kanazawa K. Antioxidative bioavailability of artepillin C in Brazilian propolis. Arch Biochem Biophys. 2004; 424(2): 181–188. doi: 10.1016/j.abb.2004.02.021 PMID: 15047190

132. Carvalho AA, Finger D, Machado CS, Schmidt EM, Costa PM, Alves APNN, et al. In vivo antitumoural activity and composition of an oil extract of Brazilian propolis. Food Chem. 2011; 126(3): 1239–1245. doi: 10.1016/j.foodchem.2010.12.035

133. Kimoto T, Aga M, Hino K, Koya-Miyata S, Yamamoto Y, Micallef MJ, et al. Apoptosis of human leukemia cells induced by Artepillin C, an active ingredient of Brazilian propolis. Anticancer Res. 2001; 21 (1A): 221–228. PMID: 11299738.

134. Matsuno T, Jung SK, Matsumoto Y, Saito M, Morikawa J. Preferential cytotoxicity to tumor cells of 3,5-diprenyl-4-hydroxycinnamic acid (Artepillin C) isolated from própolis. Anticancer. 1997; 17: 3565–3568. PMID: 9413203.

135. Kimoto T, Koya-Miyata S, Hino K, Micallef MJ, Hanaya T, Arai S, et al. Pulmonary carcinogenesis induced by ferric nitrilotriacetate in mice and protection from it by Brazilian propolis and artepillin C. Virchows Arch. 2001; 438(3): 259–270. doi: 10.1007/s004280000350 PMID: 11315623.

136. Akao Y, Maruyama H, Matsumoto K, Ohguchi K, Nishizawa K, Sakamoto T. Cell growth inhibitory effect of cinnamic acid derivatives from propolis on human tumor cell lines. Biol. Pharm. Bull. 2003; 26(7): 1057–1059. doi: 10.1248/bpb.26.1057 PMID: 12843641

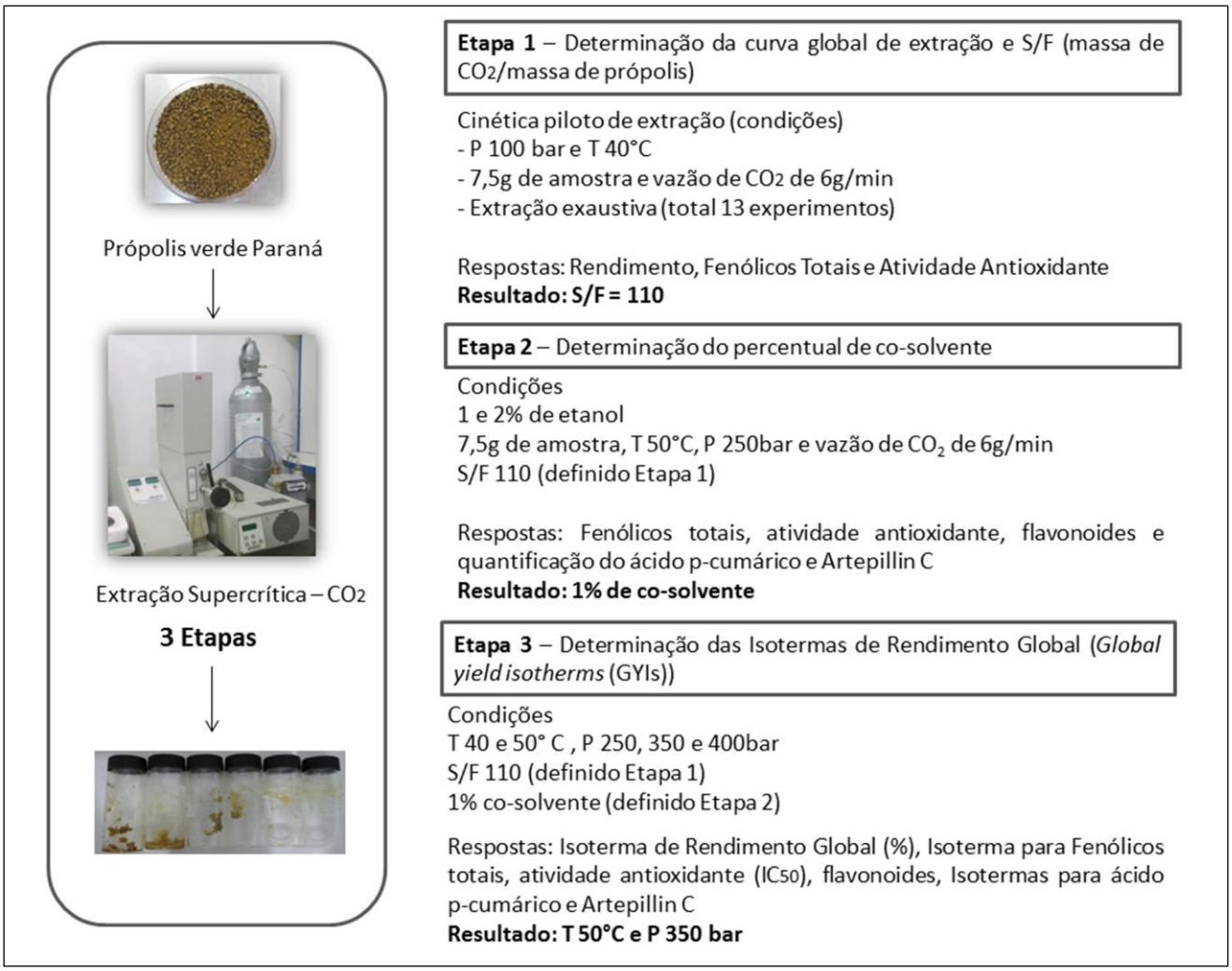
Capítulo 7. Considerações Finais

8.0 CONSIDERAÇÕES FINAIS

Neste estudo foi possível obter extratos de própolis verde com elevado teor de Artepillin C e ácido p-cumárico e foram apresentados aspectos importantes no que diz respeito aos parâmetros de processo utilizando CO_2 supercrítico como fluido extrator. Destaca-se que um dos principais aspectos que devem ser considerados na SFE é a escolha das condições operacionais no processo de extração. O uso dos valores otimizados para as diferentes condições pode melhorar significativamente o rendimento e a recuperação de um composto alvo. Além disso, o processo de extração de compostos ativos da própolis foi facilitada pela adição de pequena quantidade de co-solvente (ou modificador), o etanol, que alterou o poder de solvatação do CO_2 supercrítico. Essa flexibilidade permite a adequação de condições de extração para as necessidades específicas dos produtos a serem extraídos e ao produto final desejado. Considerando-se um material complexo como a própolis, o uso da extração com fluidos supercríticos é bastante atraente para a obtenção de extratos de alto valor agregado devido principalmente a seletividade do processo, apesar do baixo rendimento quando comparado a outros métodos convencionais (de baixa pressão) (Quadro 1).

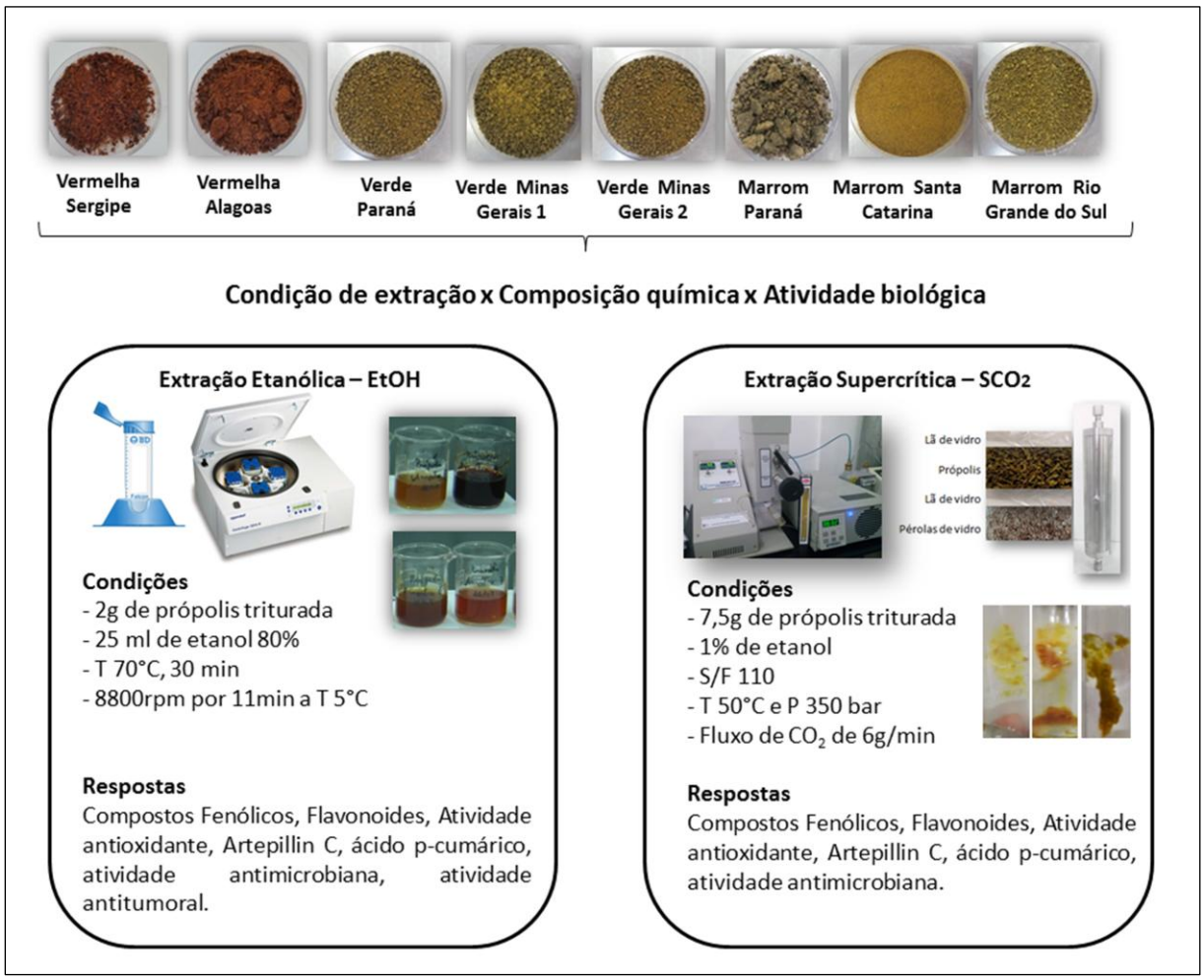
Outro ponto importante observado é que o conhecimento da origem geográfica e tipo de extração sobre as propriedades químicas e biológicas da própolis contribuem para a caracterização e padronização deste produto natural, que pode ser uma importante fonte de compostos funcionais para aplicação nas indústrias farmacêutica e alimentícia. Em suma, verificaram-se variações significativas dos componentes químicos analisados, bem como, da atividade biológica da própolis brasileira, quando se avaliou comparativamente os diferentes tipos de própolis e os diferentes métodos de extração (Quadro 2). Estas atividades biológicas são atribuídas a compostos como ácidos fenólicos, flavonoides, terpenos e sesquiterpenos, sendo o processo de extração alcoólica o mais utilizado para a obtenção desses biocompostos. Foi

confirmada a presença de Artepillin C em todas as amostras de própolis verde brasileira.



Quadro 1. Resumo da definição das condições de processo para obtenção de extratos de própolis verde utilizando CO_2 como fluido supercrítico, análises realizadas e parâmetros obtidos.

De forma geral, os extratos obtidos por extração etanólica apresentaram teores mais elevados dos compostos antioxidantes, bem como, exibiram uma maior atividade antimicrobiana quando comparado aos extratos obtidos por SFE. A SFE utilizando CO_2 como fluido supercrítico e etanol como co-solvente, pode ser utilizado para a obtenção de extratos de própolis enriquecidos em componentes específicos, tendo em vista que os teores de Artepillin C e ácido p-cumárico estiveram presentes em maior concentração nos extratos obtidos por SFE.



Quadro 2. Resumo da definição das condições de processo para obtenção de extratos de própolis de diferentes tipos pelos dois métodos de extração e análises realizadas.

Este estudo também confirmou o elevado potencial biológico apresentado pela própolis brasileira, principalmente para a própolis vermelha, que apresentou os melhores teores de compostos e atividade antioxidante e melhor propriedade biológica, incluindo a atividade antimicrobiana e atividade antiproliferativa frente a linhagens de células B16F10. Destaca-se que a própolis vermelha de Alagoas, recentemente obteve a indicação geográfica (IG) pelo Instituto Nacional da Propriedade Industrial (INPI), e é a única própolis vermelha de origem certificada no Brasil. Entretanto, neste estudo identificou-se que a própolis de Sergipe apresentou maior potencial biológico que a própolis de Alagoas, necessitando, portanto de uma organização por parte dos produtores da região para a solicitação de certificação junto ao INPI, tendo em vista a qualidade do produto.

Por fim, destaca-se que devido às suas propriedades valiosas e elevado potencial biológico, também já evidenciado em outros estudos, a própolis pode ser considerada como uma importante fonte de compostos antioxidantes naturais. Novos estudos sobre sua completa composição química estão em andamento.

Capítulo 8. Produção Técnica e Científica

Patente

1. MACHADO, B. A. S.; PADILHA, F. F.; NUNES, S. B.; GUEDES, C. M. C.; COSTA, A. S.; UMSZA-GUEZ, M. A.; SILVA, R. P. D. Patente: Patente de Invenção. Número do registro: BR1020140320121, data de depósito: 19/12/2014, título: "Processo para obtenção de ácido 3,5-diprenyl-4-hidroxicinâmico (Artepelin C) em extrato de própolis verde utilizando dióxido de carbono supercrítico e co-solvente". Instituição de registro: INPI - Instituto Nacional da Propriedade Industrial.

Artigos

1. MACHADO, B. A. S.; R. P. D.; SILVA, D. F.; BARRETO, G. A.; COSTA, S. S.; SILVA, BRANDÃO, H. N.; ROCHA, J. L. C.; UMSZA-GUEZ, M. A.; PADILHA, F. F. Chemical composition and biological activity of extracts obtained by supercritical extraction and ethanolic extraction of brown, green and red propolis derived from different geographic regions in Brazil. PLoS ONE 11(1): e0145954, 2016.
2. MACHADO, B. A. S.; BARRETO, G. A.; COSTA, A. S.; COSTA, S. S.; SILVA, R. P. D.; SILVA, D. F.; BRANDÃO, H. N.; ROCHA, J. L. C.; NUNES, S. B.; UMSZA-GUEZ, M. A.; PADILHA, F. F. Determination of parameters for the supercritical extraction of antioxidant compounds from green propolis using carbon dioxide and ethanol as co-solvent. PLoS ONE 10(8): e0134489, 2015.
3. MACHADO, B. A. S.; COSTA, A. S.; COSTA, S. S.; ALVES, A. R. C.; GUARIEIRO, L. L. N.; PADILHA, F. F. Uso de indicadores de patentes como metodologia para avaliação do desenvolvimento da tecnologia de extração supercrítica. Revista Virtual de Química. 2015. Submetido.
4. MACHADO, B. A. S.; PEREIRA, C. G.; NUNES, S. B.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives. Separation Science and Technology, v. 48, p. 2741-2760, 2013.

5. COSTA, A. S.; MACHADO, B. A. S.; GUEZ, M. A.; CIRQUEIRA, M. G.; NUNES, S. B.; PADILHA, F. F. Levantamento dos estudos realizados com a própolis produzida no estado da Bahia. Sitientibus serie Ciências Biológicas (SCB), v. 13, p. 1-7, 2013.
6. MACHADO, B. A. S.; CRUZ, L. S; NUNES, S. B.; UMSZA-GUEZ, M. A.; PADILHA, F. F. Estudo prospectivo da própolis e tecnologias correlatas sob o enfoque em documentos de patentes depositados no Brasil. GEINTEC - Gestão, Inovação e Tecnologias, v. 2, p. 221-235, 2012.

Capítulos de Livros

1. MACHADO, B. A. S.; PADILHA, F. F. O processo de proteção das Indicações Geográficas no Brasil. In: Ferlando Lima Santos. (Org.). Desenvolvimento e perspectivas da propriedade intelectual no Brasil. 1ed. Cruz das Almas: Editora UFRB, 2014, v. 1, p. 105-120.

Prêmios

1. Prêmio Melhor Trabalho Publicado Categoria 1: "Processo para obtenção de ácido 3,5-diprenyl-4-hidroxicinâmico (Artepelin C) em extrato de própolis verde utilizando dióxido de carbono supercrítico e co-solvente" - Edital PubliTec SENAI 2015, Serviço Nacional de Aprendizagem Industrial - DR Bahia. 2015.
2. Prêmio Melhor Trabalho Publicado Categoria 2: Determinación de los parâmetros de extracción de compuestos biologicamente activos del propóleo verde utilizando extracción supercrítica con CO_2" - Edital PubliTec SENAI 2015, Serviço Nacional de Aprendizagem Industrial - DR Bahia. 2015.
3. Prêmio Melhor Artigo Publicado Categoria 1: “Supercritical Fluid Extraction Using CO_2: Main Applications and Future Perspectives” - Edital PubliTec SENAI 2014, Serviço Nacional de Aprendizagem Industrial - DR Bahia. 2014
4. Diploma de Honra ao Mérito para melhor trabalho científico da área de Físico-química – “Determinação dos compostos antioxidantes e atividade

antimicrobiana de diferentes tipos de própolis brasileira" – XVIII ENAAL e IV Congresso Latino Americano de Analistas de Alimentos. 2013

Resumos Completos

1. BARRETO, G. A.; MACHADO, B. A. S.; PADILHA, F. F.. Caracterização físico-química e biológica de extratos de própolis verde e vermelha obtidos por extração com fluido supercrítico e extração etanólica. In: IV Workshop de Pesquisa Tecnologia e Inovação, 2014, Salvador. Desafios e Oportunidades para o desenvolvimento Industrial no Estado da Bahia, 2014.
2. MACHADO, B. A. S.; COSTA, A. S.; BRAMONT, W. B.; RAMOS, B. F. M.; ALVES, A. R. C.; REIS, E. C. A.; PADILHA, F. F. Determinação dos compostos antioxidantes e atividade antimicrobiana de diferentes tipos de própolis brasileira. In: XVIII ENAAL e IV Congresso Latino Americano de Analistas de Alimentos, 2013, São Paulo. Gestão e Inovação Tecnológica, 2013.

Resumos expandidos

1. COSTA, A. S.; MACHADO, B. A. S.; BRAMONT, W. B.; NUNES, S. B.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Determinação da composição centesimal de própolis verde de diferentes produtores do estado de Minas Gerais. In: II Workshop de Pesquisa, Tecnologia e Inovação, 2012, Salvador. Pesquisa, Tecnologia e Inovação, 2012.
2. MACHADO, B. A. S.; BRAMONT, W. B.; COSTA, A. S.; NUNES, S. B.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Análise comparativa do teor de compostos antioxidantes da própolis verde e marrom. In: II Workshop de Pesquisa, Tecnologia e Inovação, 2012, Salvador. Pesquisa, Tecnologia e Inovação, 2012.

Resumos

1. MACHADO, B. A. S.; BARRETO, G. A.; SILVA, R. P. D.; SILVA, D. F.; BRANDÃO, H. N.; ROCHA, J. L. C.; PADILHA, F. F.. Identificação e quantificação de Artepillin C e

Ácido p-cumárico em extratos de própolis obtidos por extração supercrítica e etanólica. In: 6º Encontro Regional da SBQ Nordeste, 2015, Maceió. A Química e a Biotecnologia do Nordeste: o caminho da Pesquisa para a Inovação, 2015.

2. SILVA, D. F.; ROCHA, J. L. C.; BARRETO, G. A.; COSTA, A. S.; MACHADO, B. A. S.; PADILHA, F. F.; BRANDÃO, H. N. Validação de metodologia para quantificação de ácido p-cumárico e Artepillin C em extratos de própolis por cromatografia a líquidos de alta eficiência com detector de arranjo de diodos. In: VII Simpósio Iberoamericano de Plantas Medicinais - II Simpósio Iberoamericano de Investigação em Câncer, 2014, Ilhéus. Plantas Medicinais, 2014.
3. MACHADO, B. A. S.; BARRETO, G. A.; SILVA, R. P. D.; UMSZA-GUEZ, M. A.; PADILHA, F. F. Determinação do teor de compostos bioativos da própolis verde de Minas Gerais e Paraná: um potencial para a proteção por indicação geográfica. In: III Simpósio Internacional de Indicações Geográficas, 2014, Ilhéus. Indicações Geográficas, 2014.
4. MACHADO, B. A. S.; COSTA, A. S.; BARRETO, G. A.; NUNES, S. B.; UMSZA-GUEZ, M. A.; COELHO, E.; PADILHA, F. F. Determinación de los parâmetros de extracción de compuestos biologicamente activos del propóleo verde utilizando extracción supercrítica con CO_2. In: do V Congreso Internacional de Ciencia y Tecnología de Alimentos, 2014, Córdoba. Ciencia y Tecnología de Alimentos, 2014.
5. MACHADO, B. A. S.; REIS, E. C. A.; COSTA, A. S.; ALVES, A. R. C.; OLIVEIRA, E. A.; PADILHA, F. F. Study of the bioactive compoinds of the supercritical extracts with and without co-solvent of red propolis. In: Nonthermal Food Processing Workshop, 2013, Florianópolis. Nonthermal Food Processing Workshop, 2013.
6. MACHADO, B. A. S.; NUNES, S. B.; SILVA, C. H. M.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Comparative study of brown propolis obtained from different regions of Brazil. In: XVI World Congress of Food Science and Technology - IUFOST, 2012, Foz do Iguaçu. Food Science and Technology, 2012.
7. MACHADO, B. A. S.; NUNES, S. B.; SILVA, C. H. M.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Determination of phenolic compounds in of ethanolic extracts green propolis native to the Brazilian state of Minas Gerais. In: XVI World Congress of

Food Science and Technology - IUFOST, 2012, Foz do Iguaçu. Food Science and Technology, 2012.

8. MACHADO, B. A. S.; NUNES, S. B.; NUNES, I. L.; PADILHA, F. F.; UMSZA-GUEZ, M. A. Avaliação do teor de compostos fenólicos da própolis verde de Minas Gerais: um potencial produto para a proteção por Indicação Geográfica. In: II Simpósio Internacional de Indicações Geográficas, 2012, Fortaleza. Indicações Geográficas, 2012.

Anexo 1

Tabela 1. Cinética piloto com valores de CO_2 em massa (Kg) e volume (m^3) utilizado em cada experimento.

Número do experimento	Tempo (min)	Volume de CO_2 (m^3)	Volume de CO_2 (m^3) Acumulado	S/F (massa de CO_2/Massa de própolis)	Massa de CO_2 (Kg)	Massa de CO_2 (Kg) Acumulado
1	8,68	0,030	0,030	7,28	0,055	0,055
2	18,39	0,030	0,060	14,56	0,055	0,109
3	29,43	0,030	0,090	21,84	0,055	0,164
4	45,68	0,045	0,135	32,76	0,082	0,246
5	58,68	0,045	0,180	43,68	0,082	0,328
6	78,50	0,060	0,240	58,25	0,109	0,437
7	100,50	0,060	0,300	72,81	0,109	0,546
8	121,50	0,060	0,360	87,37	0,109	0,655
9	150,50	0,090	0,450	109,22	0,164	0,819
10	187,50	0,090	0,540	131,06	0,164	0,983
11	224,50	0,090	0,630	152,91	0,164	1,147
12	252,50	0,090	0,720	174,75	0,164	1,311
13	288,50	0,090	0,810	196,60	0,164	1,475

Anexo 2

Metodologia detalhada para a identificação e quantificação de compostos fenólicos em extratos de própolis por Cromatografia Líquida de Alta Eficiência (HPLC)

Preparo das amostras

O preparo da amostra foi realizado de acordo com Daugsch (2009), em que foram preparadas soluções de 10 mg/ml dos extratos. Para os extratos obtidos por extração etanólica, as amostras foram dissolvidas em metanol, enquanto que para os extratos obtidos por extração com fluido supercrítico, os extratos foram dissolvidos em etanol e colocados em banho ultrassom por 30 minutos. As amostras foram filtradas em filtro de membrana de éster de celulose 0,45 µm (Micropore®) para então ser injetado no HPLC (Cromatografia Líquida de Alta Eficiência).

Reagentes

As quantificações dos fenólicos envolveram o uso dos padrões de Kaempferide, Galato de Propila, Ácido Felúrico, Ácido *p*-cumárico, Rutina e Formononetina da Sigma-Aldrich e Artepillin C da Wako Pure Chemical Industries, Ltd. (Osaka, Japan). Para as análises em HPLC foram usados solventes de grau cromatográfico de pureza (Merck®) e água purificada em sistema Milli-Q®.

Análises Cromatográficas

Os experimentos cromatográficos foram realizados com sistema HPLC EZChrom Elite, consistindo de bomba VRW HITACHI L-2130, equipado com injetor automático e detector de arranjo de diodo (DAD) VRW HITACHI L-2455 e forno VRW HITACHI L-2300. A separação cromatográfica foi baseada no método de Daugsch (2009) com adaptações. Foi utilizada coluna LiChroCART Purospher StaR® RP18-e (75 mm x 4 mm i.d.) (3µm) (Merck, Darmastad, Germany) combinado com pré-coluna LiChroCART 4-4 LiChrospher 100RP18 (5µm) da Merck. As condições de análise foram realizadas com gradiente de eluição conduzido com fase móvel de ácido acético 5% (fase aquosa) e

metanol (fase orgânica) em diferentes proporções de acordo com a Tabela 1. O volume de injeção foi de 10 µL. O equipamento foi operado à temperatura ambiente (25±2 °C).

Tabela 1. Sistema de gradiente da fase móvel

Tempo (min:seg)	Solução de ácido acético 5% (%)	Metanol (%)
0:00	80	20
10:00	70	30
15:00	60	40
30:00	50	50
45:00	40	60
65:00	30	70
65:01	0	100
70:00	80	20

A leitura do detector de arranjo de diodo foi na faixa de 200 a 400 nm e a aquisição cromatográfica definida em 290 nm. A identificação dos compostos foi conduzida pela comparação dos tempos de retenção e do espectro de ultravioleta das amostras com os padrões.

Identificação e quantificação dos padrões (fenólicos)

As amostras foram analisadas para a identificação dos, sendo utilizados o Kaempferide, Galato de Propila, Ácido Felúrico, Ácido *p*-cumárico, Rutina, Formononetina e Artepillin C, comparando os picos e espectros de UV obtidos com os dos padrões.

Para a quantificação foram utilizados apenas os fenólicos encontrados nas amostras, sendo esses o Ácido *p*-cumárico e Artepillin C. A quantificação foi realizada utilizando-se as curvas dos padrões, interpolando o valor de área das amostras encontradas, nas equações da reta obtidas.

Validação

A validação foi realizada segundo parâmetros da Agência Nacional de Vigilância Sanitária (ANVISA) – Resolução ANVISA RE n° 899, de 29/05/2003 – e pelo Instituto Nacional de Metrologia, Normalização e Qualidade Industrial (INMETRO) – documento INMETRO DOQ-CGCRE-008, de março/2003. O método analítico foi validado de acordo com os parâmetros de seletividade, linearidade, precisão, exatidão, limite de detecção e limite de quantificação.

A seletividade foi determinada por meio da comparação de picos dos padrões com os picos correspondentes nas amostras, levando-se em consideração: tempo de retenção e espectro de ultravioleta (UV).

A linearidade foi determinada pela curva de calibração, levando-se em consideração o coeficiente de correlação (R^2). As curvas de calibração foram obtidas por injeções de soluções com sete diferentes concentrações dos padrões externos, numa faixa de 37 a 1000 µg/ml para o Artepillin C e de 3,4 a 340 µg/ml para o ácido *p*-cumárico. As soluções de cada padrão externo foram preparadas pela dissolução do padrão em metanol, que representa a fase móvel do método analítico, em balão volumétrico.

A precisão foi determinada pela injeção em triplicata de três soluções de cada um dos padrões. Esse parâmetro foi expresso como o desvio padrão relativo, segundo a fórmula demonstrada na Equação 1.

$$\mathrm{DPR} = \frac{\mathrm{DP}}{\mathrm{CMD}} \mathrm{x}100$$

(Equação 1)

Em que, DP é o desvio padrão e CMD, a concentração média determinada.

A exatidão foi verificada pelo fator de recuperação. Amostras de própolis foram fortificadas com três soluções padrão de concentrações conhecidas de 57, 76 e 91 µg/ml para o ácido *p*-cumárico e 163, 193 e 243 µg/ml para o Artepillin C. As amostras fortificadas, juntamente com amostra sem fortificação (branco), foram submetidas à injeção em HPLC. A exatidão foi avaliada através de valores de concentração

determinados experimentalmente, comparados à concentração teórica como pode ser verificado na Equação 2.

$$Rec\% = \frac{[\text{valor obtido - valor real}]}{\text{valor real}} x100$$

(Equação 2)

O limite de detecção (LD) foi estimado pela relação do desvio padrão e da inclinação da curva de calibração, conforme apresentado na Equação 3.

$$LD = \frac{DP_a \, x3}{IC}$$

(Equação 3)

Em que, DPa é o desvio padrão obtido a partir da curva de calibração; IC é a inclinação da curva de calibração.

O limite de quantificação (LQ) foi estimado pela relação do desvio padrão e da inclinação da curva de calibração, segundo a Equação 4:

$$LQ = \frac{DP_a x10}{IC}$$

(Equação 4)

Em que, DPa é o desvio padrão obtido a partir da curva de calibração; IC é a inclinação da curva de calibração.

Resultados

Através da comparação com os tempos de retenção e dos espectros de Ultravioleta (UV) dos padrões e das amostras, realizou-se a análise qualitativa dos extratos de própolis, identificando a presença do ácido *p*-cumárico e Artepillin C. No entanto, os demais padrões não foram detectados nos extratos nas condições analisadas, contudo, não se pode afirmar a ausência dos mesmos.

Os cromatogramas e espectros de UV dos padrões estão representados nas figuras a seguir: Ácido *p*-cumárico (Figura 1 e Figura 2), Ácido Felúrico (Figura 3 e

Figura 4), Galato de Propila (Figura 5 e Figura 6), Rutina (Figura 7 e Figura 8), Formononetina (Figura 9 e Figura 10), Kaempferide (Figura 11 e Figura 12) e Artepillin C (Figura 13 e Figura 14).

As Figuras 15 e 16 mostram os cromatogramas ilustrativos das amostras, sendo que a Figura 15 mostra o cromatograma da amostra Própolis Vermelha SE (extração com etanol), a qual não apresentou nenhum dos padrões analisados, enquanto que a Figura 16 mostra o cromatograma da Própolis Verde PR (extração com etanol), no qual foram identificados do ácido *p*-cumárico e Artepillin C.

Figura 1. Cromatograma do padrão Ácido *p*-cumárico a 290 nm em HPLC-DAD

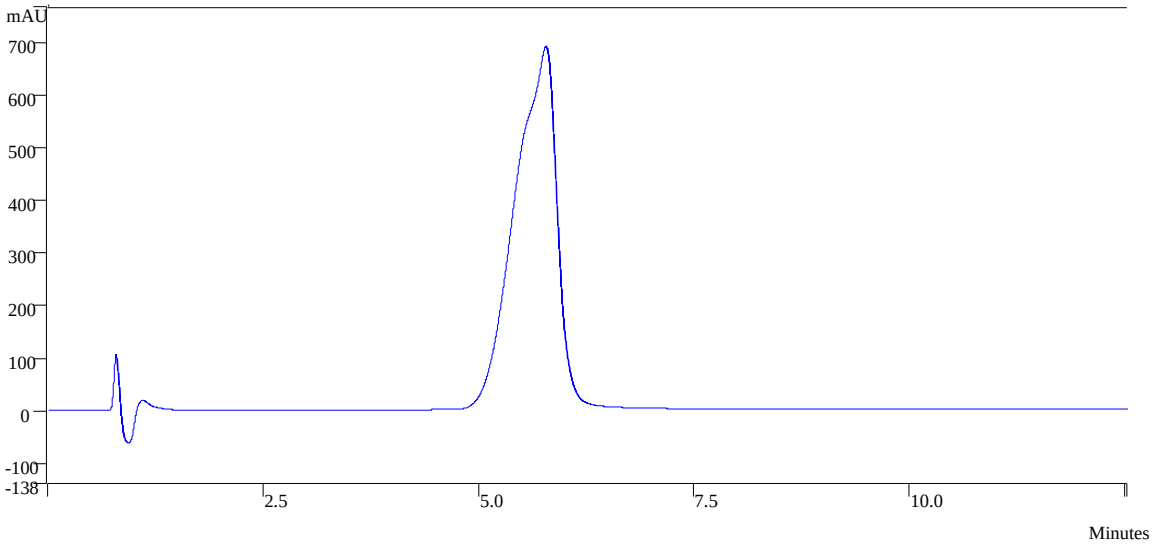


Figura 2. Espectro de UV do padrão de Ácido *p*-cumárico a 290 nm em HPLC-DAD

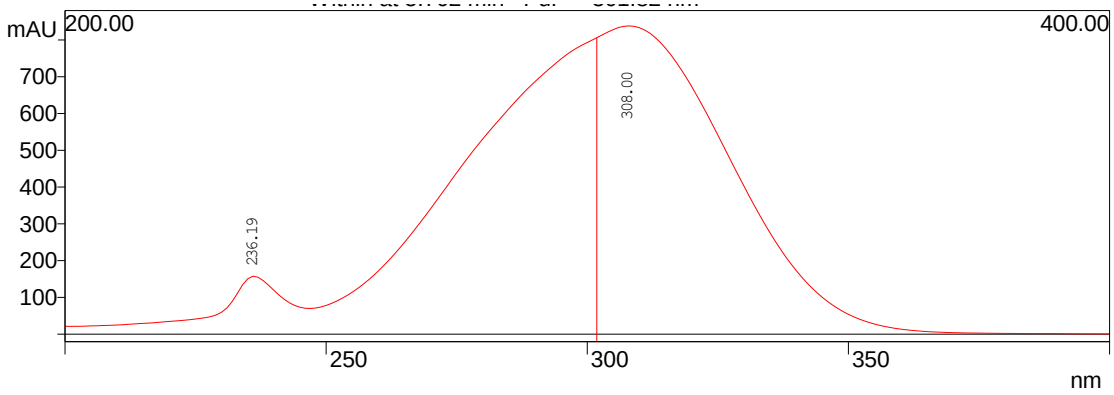


Figura 3. Cromatograma do padrão Ácido Felúrico a 290 nm em HPLC-DAD

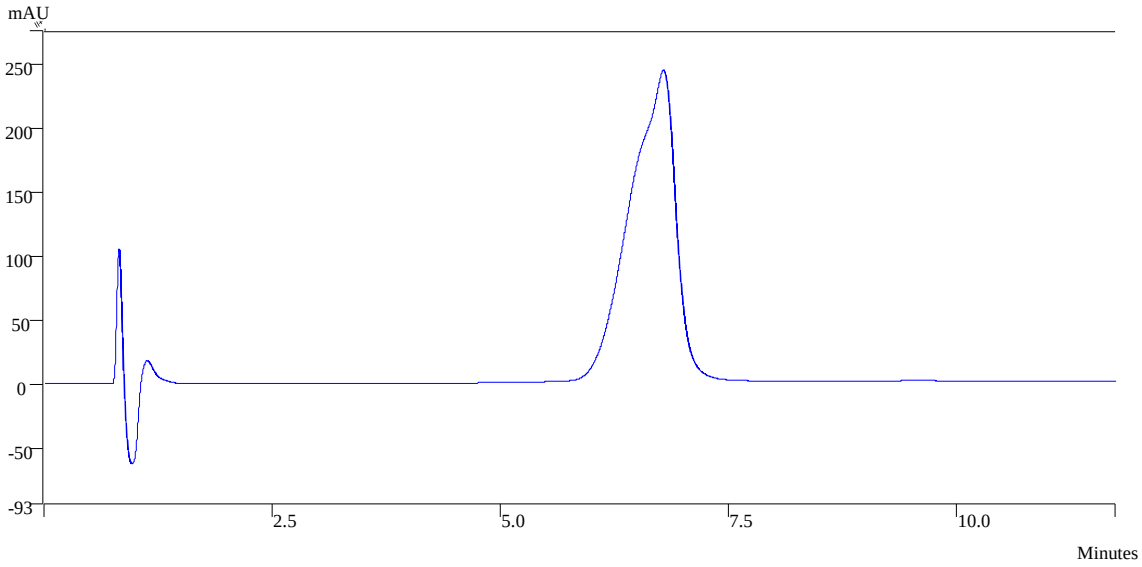


Figura 4. Espectro de UV do padrão de Ácido Felúrico a 290 nm em HPLC-DAD

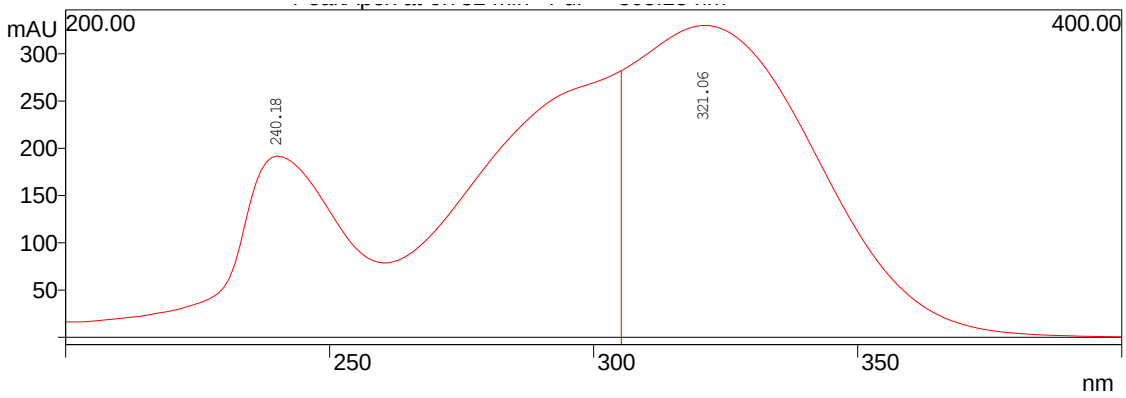


Figura 5. Cromatograma do padrão de Galato de Propila a 290 nm em CLAE-DAD a 290 nm em HPLC-DAD

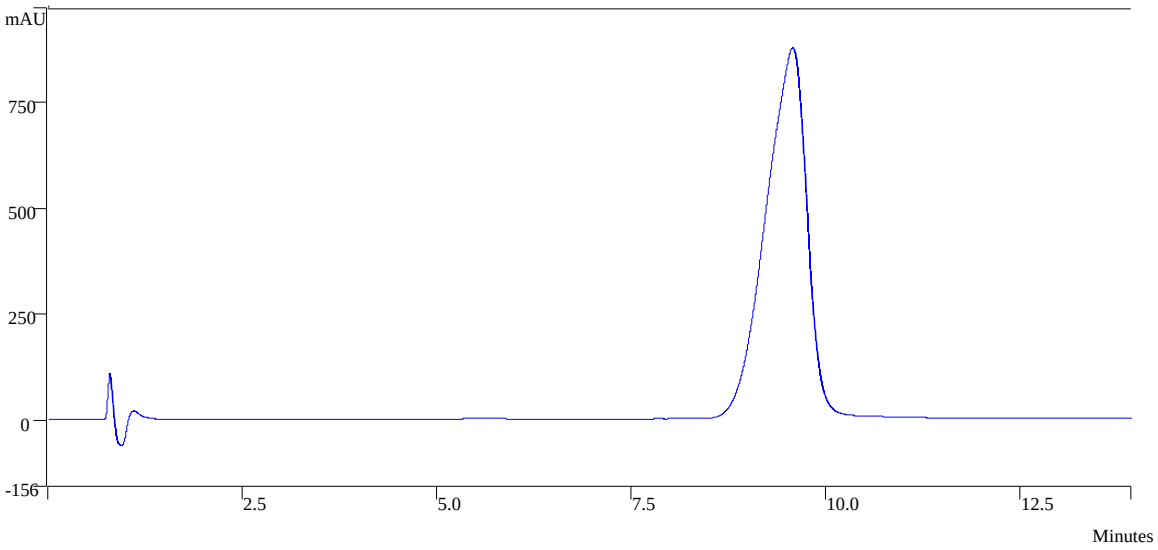


Figura 6. Espectro de UV do padrão de Galato de Propila a 290 nm em HPLC-DAD

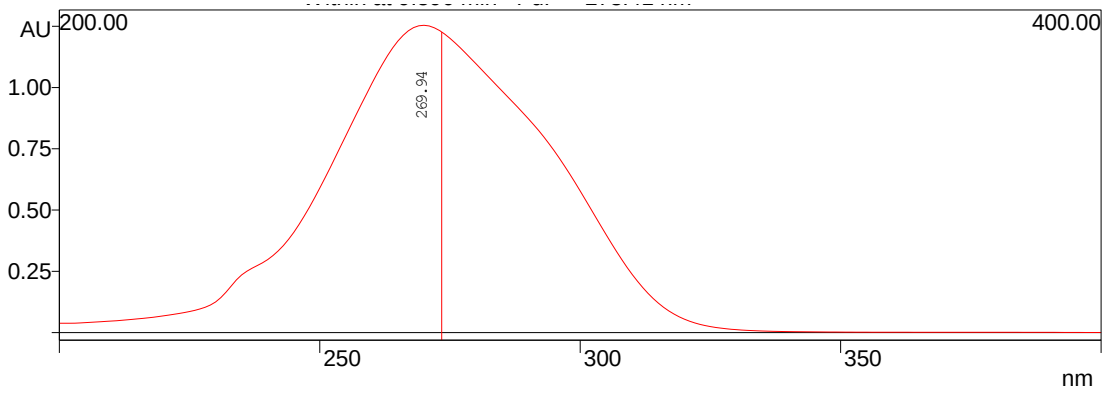


Figura 7. Cromatograma do padrão de Rutina a 290 nm em HPLC-DAD

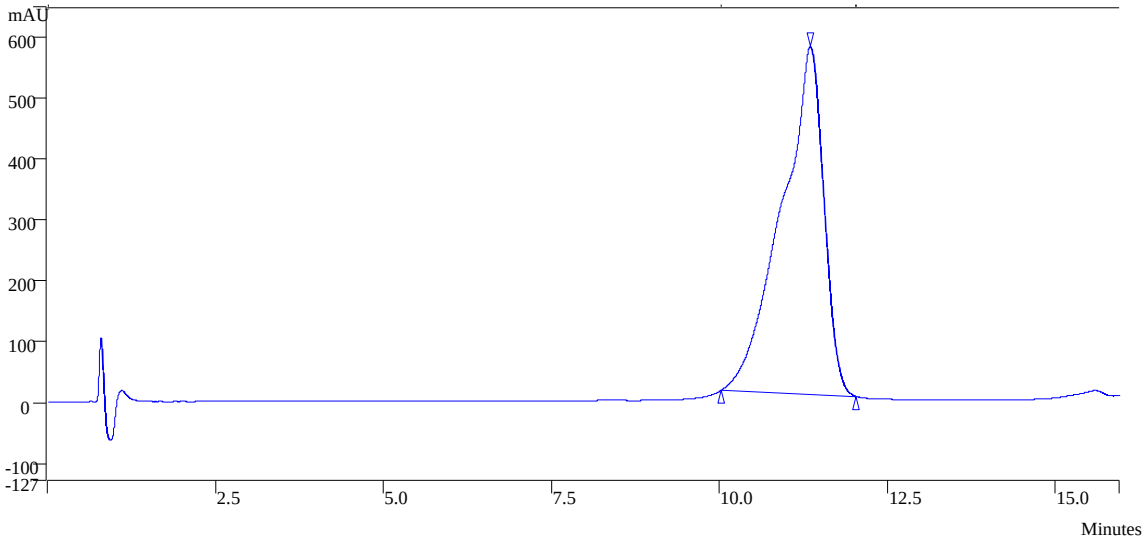


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Figura 8. Espectro de UV do padrão de Rutina a 290 nm em HPLC-DAD

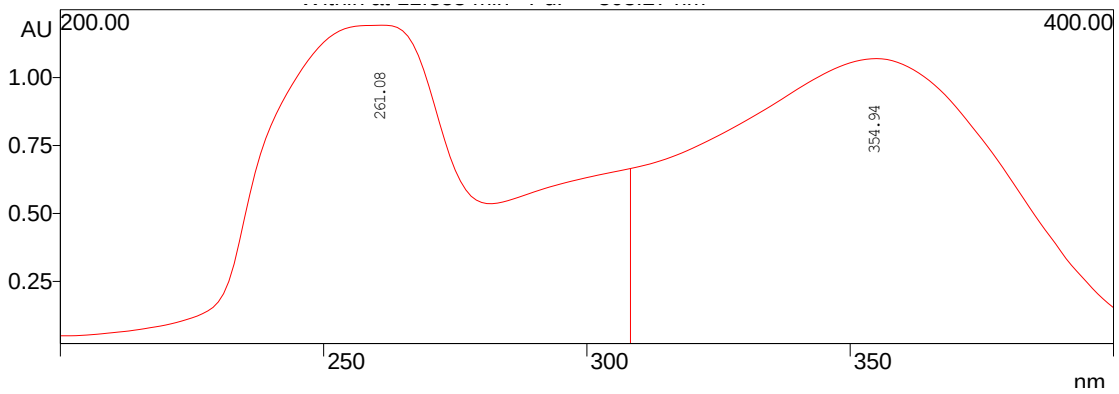


Figura 9. Cromatograma do padrão de Formononetina a 290 nm em HPLC-DAD

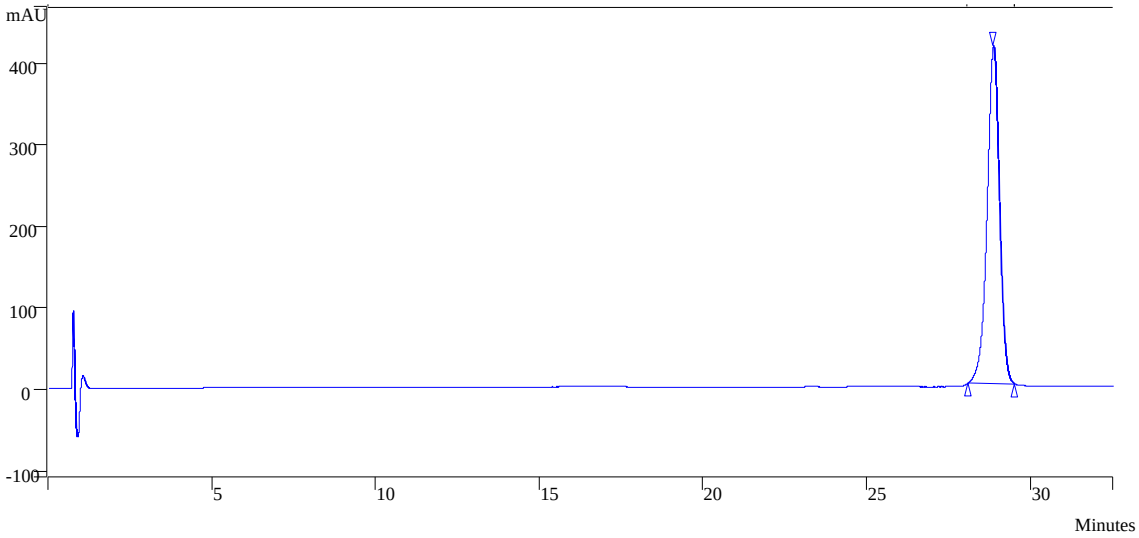


Figura 10. Espectro de UV do padrão de Formononetina a 290 nm em HPLC-DAD

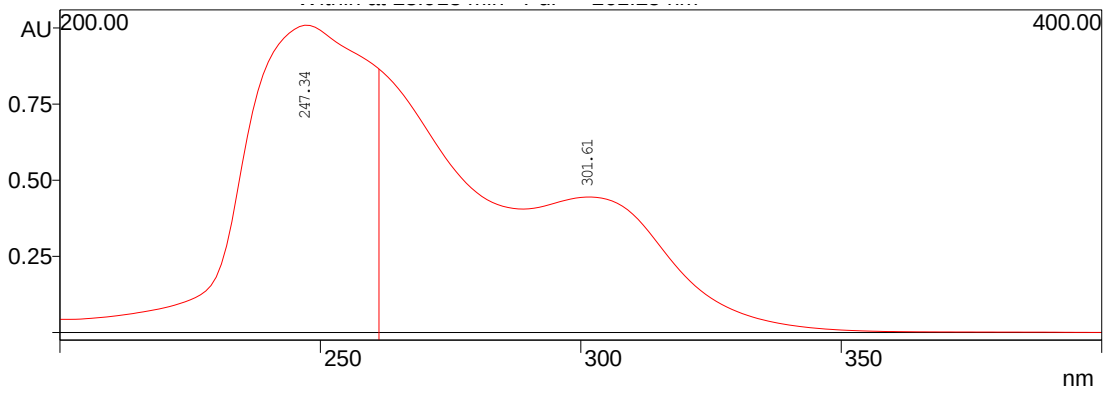


Figura 11. Cromatograma do padrão de Kaempferide a 290 nm em HPLC-DAD

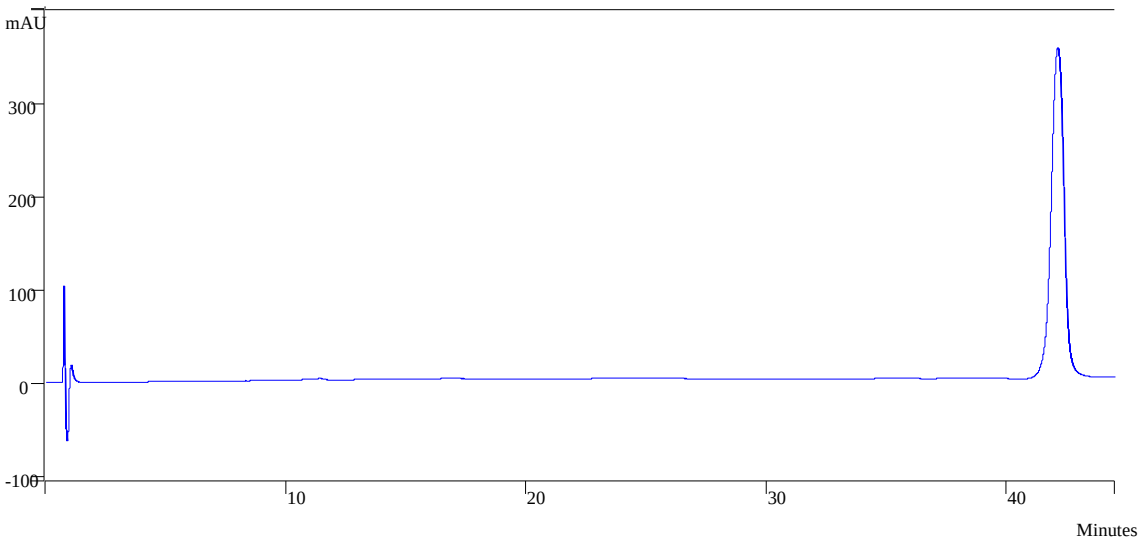


Figura 12. Espectro de UV do padrão de Kaempferide a 290 nm em HPLC-DAD

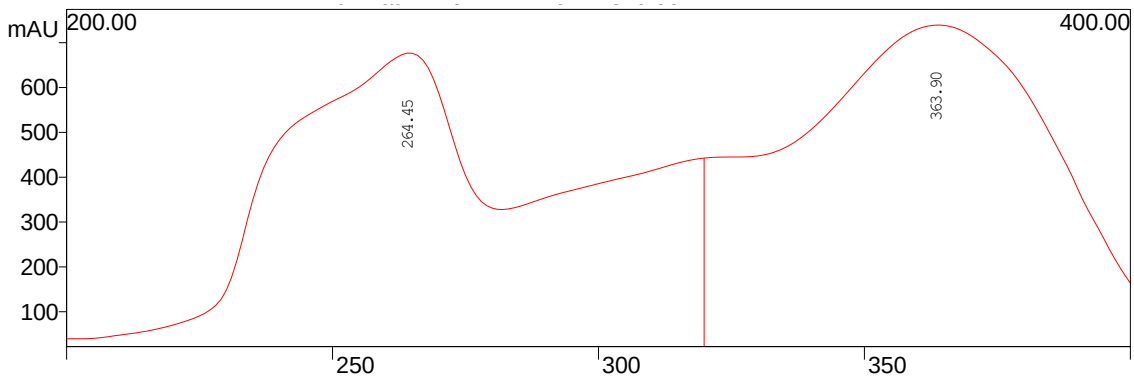


Figura 13. Cromatograma do padrão de Artepillin C a 290 nm em HPLC-DAD

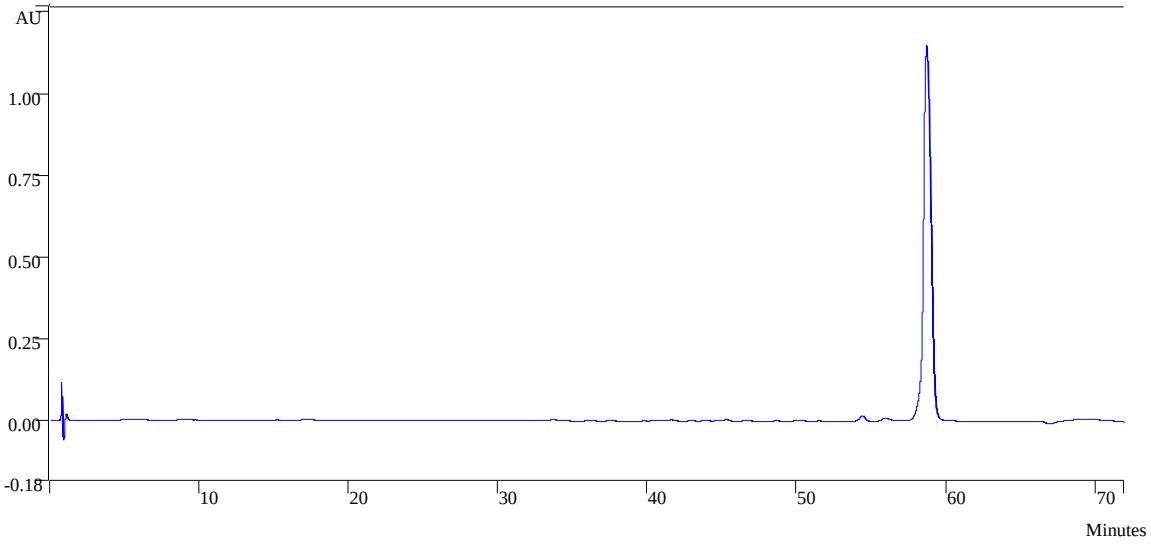


Figura 14. Espectro de UV do padrão de Artepillin C a 290 nm em HPLC-DAD

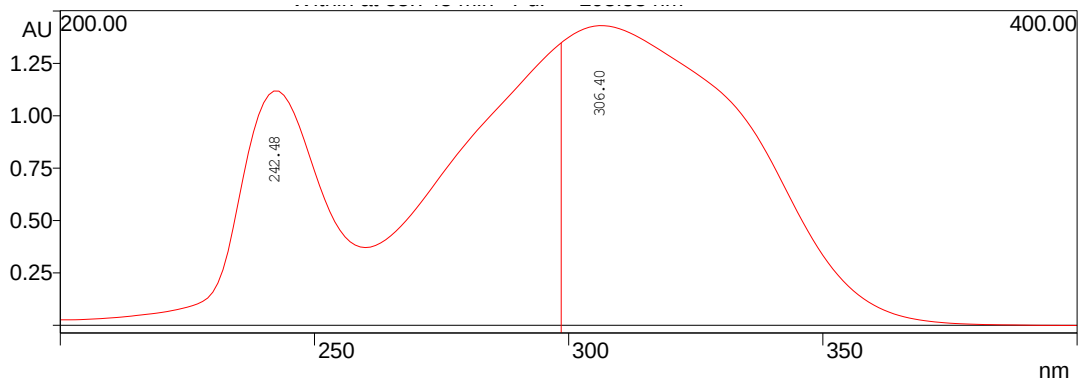


Figura 15. Cromatograma da amostra Própolis Vermelha SE (extrato etanólico) a 290 nm em HPLC-DAD

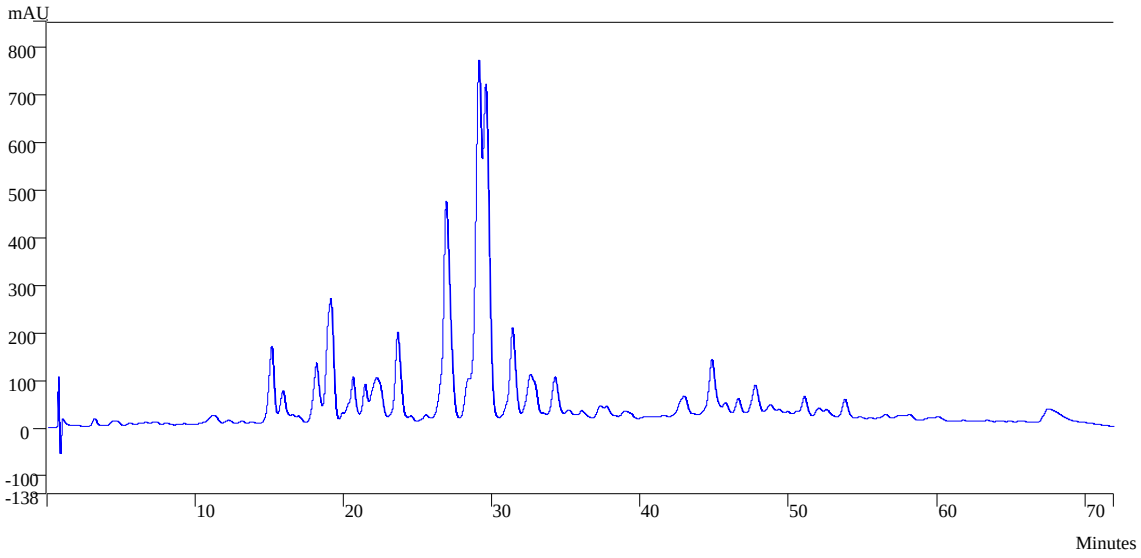
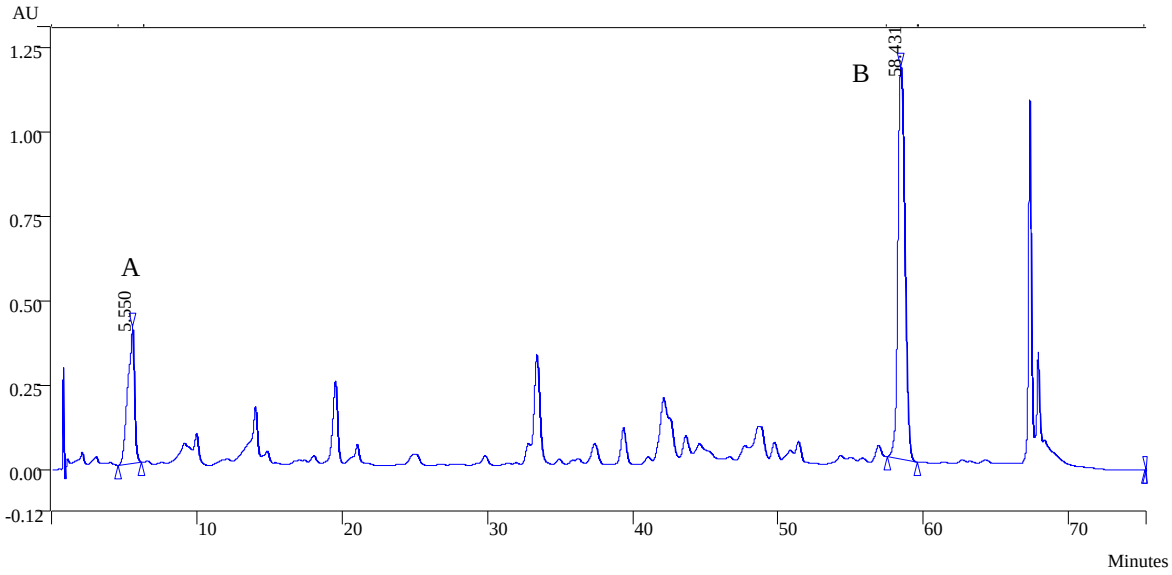


Figura 16. Cromatograma da amostra Própolis Verde PR (Extrato etanólico) a 290 nm em HPLC (A) Ácido *p*-cumárico, (B) Artepillin C.



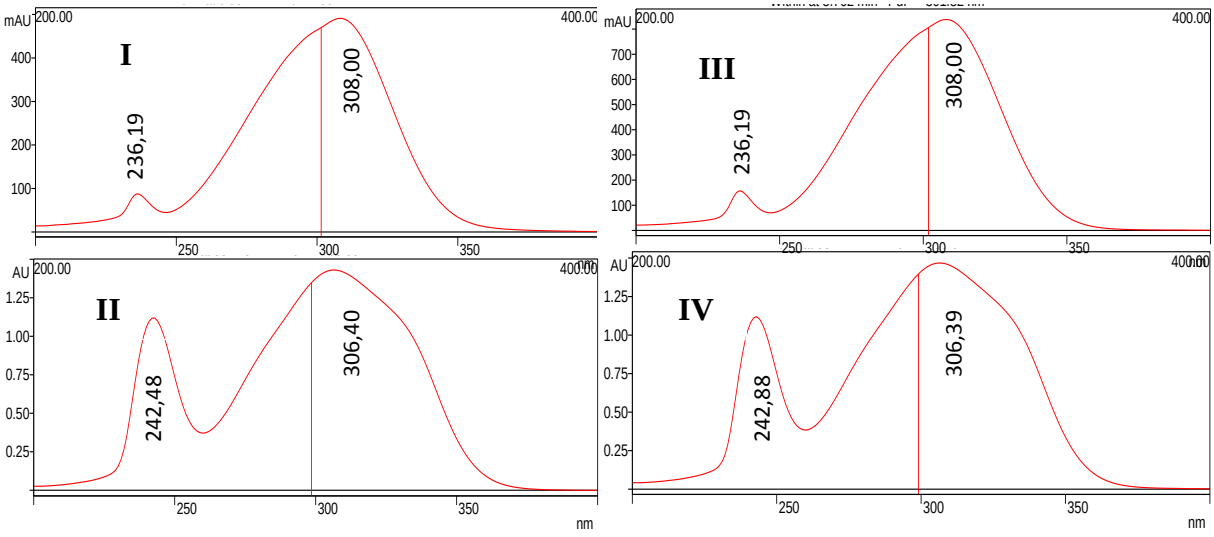
A seletividade foi avaliada através da comparação dos cromatogramas dos padrões e amostras, pela avaliação dos tempos de retenção. A Tabela 2 apresenta os tempos de retenção das substâncias analisadas nos extratos e dos padrões de referência.

Tabela 2. Tempo de retenção dos padrões e da amostra.

	Pico	Tempo de retenção (min)
Amostra	A	5,550
	B	58,431
Padrão	Ácido *p*-cumárico	5,762
	Artepillin C	58,745

Corroborando com essa análise, através da Figura 17 pode-se observar os espectros de UV dos padrões e da amostra, obtido no comprimento de onda de 290 nm, indicando que o método é seletivo tanto para o ácido *p*-cumárico como para o Artepillin C.

Figura 17 – Comparação ilustrativa dos espectros de UV de amostra e do padrão. I - padrão de ácido *p*-cumárico, II - padrão de Artepillin C, III - pico A da amostra na Figura 16; IV- pico B da amostra na Figura 16.



O método proposto foi avaliado quanto a sua linearidade por meio de regressões lineares da curva analítica. As curvas de calibração (Figuras 18 e 19) foram construídas a partir da relação entre sete concentrações de cada padrão com as áreas obtidas na separação cromatográfica. As curvas obtidas mostraram-se adequadamente

linear com coeficientes de correlação (R^2) de 0,999 para o Artepillin C e para o ácido *p*-cumárico, mantendo o valor considerado ideal para linearidade, que segundo a ANVISA é maior ou igual a 0,99 (BRASIL, 2003).

Figura 18. Curva de calibração do ácido *p*-cumárico e parâmetros obtidos com a regressão linear.

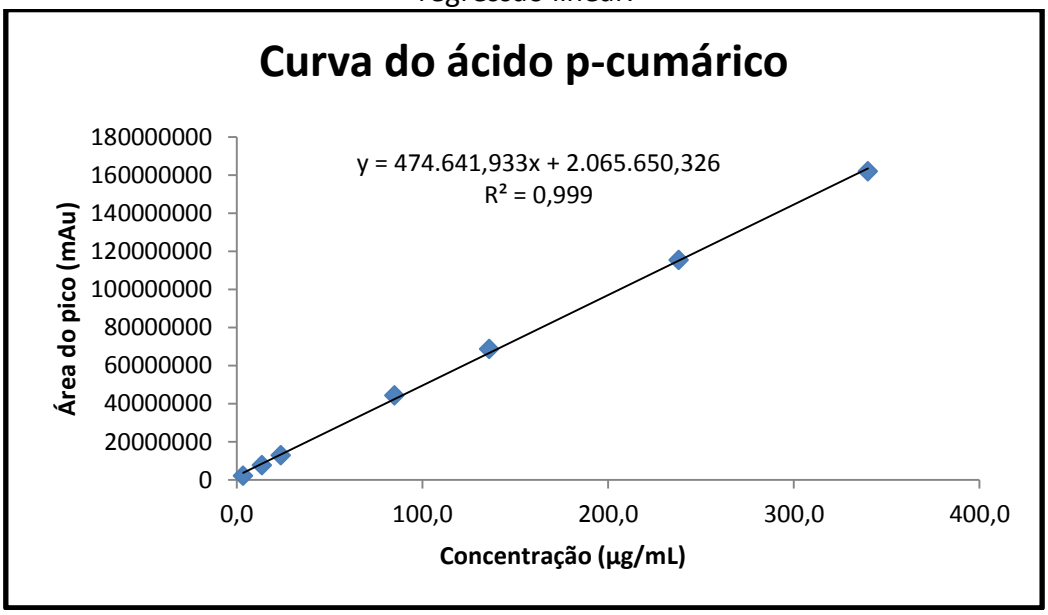
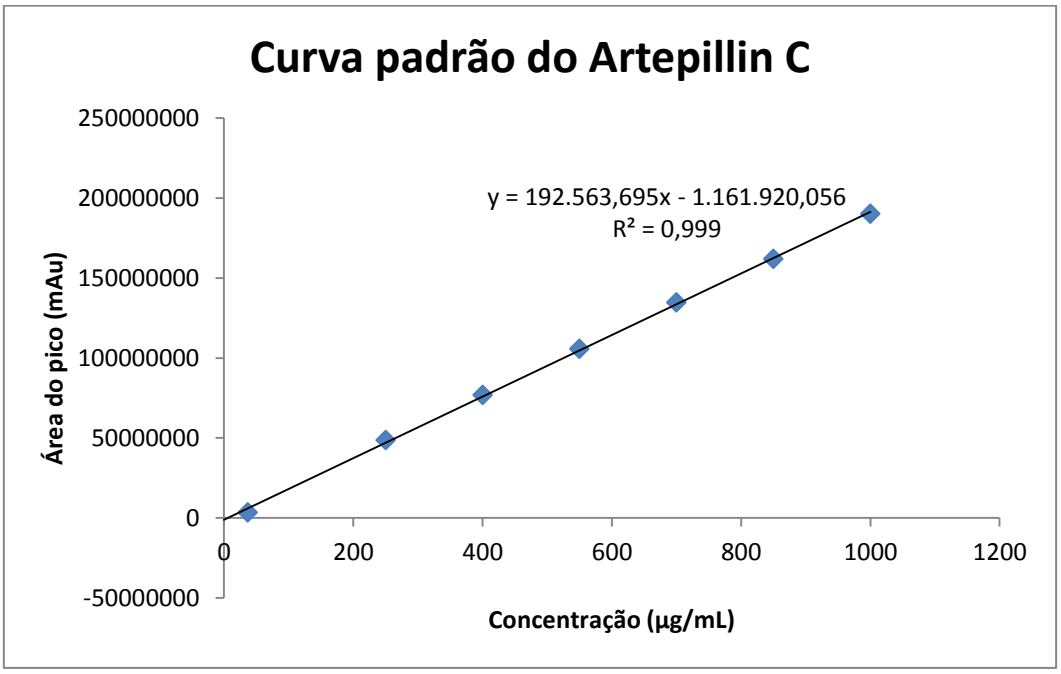


Figura 19. Curva de calibração do Artepillin C e parâmetros obtidos com a regressão linear.



A precisão do método foi determinado pelo desvio padrão relativo de três soluções padrões, injetadas em triplicata. Os resultados obtidos se encontram dentro da faixa de precisão preconizada pela ANVISA, inferior a 5% (BRASIL, 2003), conforme apresentado na Tabela 3.

Tabela 3. Dados para avaliação da precisão do método

	Média das áreas (mAu)	**Desvio padrão (mAu)**	**Desvio padrão relativo (%)**
Ácido *p*-cumárico	161896373	2501031	1,54
	44357497	140185	0,36
	2075849	21780	1,05
Artepillin C	190071643	322419	0,17
	76865779	841061	1,09
	3327349	100868	3,03

A exatidão foi avaliada por meio da porcentagem de recuperação dos padrões, através da adição de soluções dos padrões, em triplicatas, nas concentrações de 57, 76 e 91 µg/mL para o ácido *p*-cumárico e 163, 193 e 243 µg/mL para o Artepillin C. As taxas de recuperação demonstram a exatidão do método utilizado, que, como pode ser observado na Tabela 4, encontram-se dentro da faixa estabelecida pela ANVISA, que é de 80 a 120%, podendo ser considerados ainda valores de 50% a 120% para amostras complexas (RIBANI *et al.*, 2004).

Tabela 4. Dados para avaliação da exatidão do método

	Concentração adicionada (µg/mL)	**Concentração recuperada (µg/mL)**	**Exatidão (%)**
Ácido *p*-cumárico	57,00	64,70	113,51
	76,00	75,51	99,36

	91,00	94,86	104,24
Artepillin C	163,00	102,64	62,67
	193,00	140,69	72,90
	243,00	175,08	72,05

Os limites de detecção (LD) e de quantificação (LQ) foram calculados a partir das curvas de calibração dos padrões ácido *p*-cumárico e Artepillin C. Os resultados encontrados para o ácido *p*-cumárico foram de LD= 0,22 µg/ml e LQ= 0,73 µg/ml, e para o Artepillin C foram de LD= 5,02 µg/ml e LQ= 16,74 µg/ml, podendo assim inferir que o método é suficientemente sensível para os compostos de interesse.

As concentrações do ácido *p*-cumárico e Artepillin C nas amostras foram determinadas pela substituição do y das equações das curvas dos padrões, pelos valores obtidos das médias (n=3) das áreas dos picos das respectivas substâncias nas análises. Os valores obtidos para cada extrato foram apresentados nos Capítulos 5 e 6.

Anexo 3

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Protocolo

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Número do protocolo:	860140211442
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Número de referência do envio:	84484

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Referência interna:	PATALISENAI003
Primeiro requerente ou interessado:	SERVIÇO NACIONAL DE APRENDIZAGEM INDUSTRIAL
CNPJ do primeiro requerente ou interessado:	03.795.071/0001-16
Número de requerentes ou interessados:	1
Título do pedido:	PROCESSO PARA OBTENÇÃO DE ÁCIDO 3,5-DIPRENYL-4-HIDROXICINÂMICO (ARTEPELIN C) EM EXTRATO DE PRÓPOLIS VERDE UTILIZANDO DIÓXIDO DE CARBONO SUPERCRÍTICO E CO-SOLVENTE

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